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PRINCIPAL INVESTIGATOR: Michael Reiss, M.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, Connecticut 06520-8047

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Michael Reiss, M.D.

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Yale University
New Haven, Connecticut 06520-8047
E-Mail: michael.reiss@yale.edu

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Transforming Growth Factor-ß (TGFß) is the most potent known inhibitor of cell cycle progression of normal mammary epithelial cells. In general, advanced breast cancers are refractory to TGFß-mediated growth inhibition. The TGFß type I and type II receptors (TßR-I and -II) are the primary transducers of TGFß's antiproliferative effects. It is our working hypothesis that TGFß-resistance is caused by lesions in the TßR genes.

Using touch preps of primary breast carcinomas, we showed that approximately 50% of primary breast carcinomas contain subpopulations of cells that have lost one or both copies of the TßR-I or -II gene. Screening of the TßR-I and -II genes for the presence of mutations using PCR-SSCP and DNA sequencing indicate that a particular missense mutation (S387Y) in TßR-I is present in approximately 6% of primary cancers and 42% of axillary lymph node metastases. Finally, we have generated antibodies that specifically recognize the activated (phosphorylated) forms of Smad2 and -3. These allow us to assess in situ whether breast cancer cells are responding to TGFß, and whether TßR defects are present or not. Approximately 12% of primary breast cancers fail to activate Smad2, indicating the presence of a TGFß receptor defect.

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INTRODUCTION

Transforming Growth Factor-ß (TGFß) is the most potent known inhibitor of cell cycle progression of normal mammary epithelial cells; in addition, it causes cells to deposit increased amounts of extracellular matrix, which affects cell-cell and cell-substrate interactions. In general, advanced breast cancers are refractory to TGFß-mediated growth inhibition, while the TGFß they secrete apparently serves to enhance invasion into surrounding structures and perhaps their metastatic potential. The effects of TGFß on cell cycle progression are transduced by two cell surface receptors, TGFß type I (TßR-I) and -II receptors (TßR-II), and relayed from the membrane to the cell nucleus by three recently discovered members of the MAD family of proteins, Smad2, -3, and -4. It is our working hypothesis that TGFß-resistance can, in principle, be caused by molecular lesions in any of these five genes, that such lesions are likely to occur during the development or progression of human breast cancer, and that they may impact on prognosis or treatment response.

This project addresses three of the fundamental research issues raised by the USAMRMC Breast Cancer Research Program. The first question is whether or not molecular lesions of the genes involved in the TGFß signaling pathway contribute to the origin and/or progression of breast cancer. We expected changes in these genes to be relatively late events, perhaps characteristic of metastatic cancer. Secondly, we proposed to determine how molecular lesions in the TGFß receptor and/or Smad genes affect receptor function, and how they might play a role in the development and/or progression of breast cancer. Thirdly, we intended to examine the question whether genetic lesions in TGFß receptor and/or Smad genes are able to predict the outcome of patients with breast cancer. Because the anti-tumor effects of anti-estrogens such as tamoxifen are thought to be mediated by the auto- and paracrine induction of TGFß, we wished to test the hypothesis that resistance of hormone-receptor positive cancers to tamoxifen is the result of inactivation of TGFß pathway genes.

BODY

The Statement of Work in our original proposal included the following tasks/timeline:

Task 1. Screening for mutations in TGFß receptor genes in breast cancer

a. Identification of genetic alterations of TGFß-receptor genes in invasive breast cancer specimens. - Months 1-24

b. Identification of genetic alterations of TGFß-receptor gene in sets of pre-invasive, primary invasive and metastatic (lymph node positive) breast cancers in order to determine the stage of tumor development at which these mutations occur. Months 12-36.

Task 2. Determination of the functional consequences of TGFß-receptor mutations

Cloning of TGFß-receptor mutants into mammalian expression vectors and transfection into TGFß-sensitive and -resistant human mammary epithelial cells to determine whether the mutations are dominant or recessive, and correlation of the site of mutations within the molecule with the way they affect the cellular phenotype. - Months 12-36

Task 3. To determine the potential clinical significance of genetic alterations of the TßR genes in breast cancer

Test the hypothesis that genetic alterations of TGFß-receptor genes predict for resistance to anti-estrogen therapy in patients with estrogen-receptor positive tumors. Months 36-48.

Progress achieved on each of these tasks will be described separately:

Task 1. Screening for mutations in TGFß receptor genes in breast cancer

Our initial studies of genes involved in TGFß signaling focused on the TßR-II gene. Using a chemical mismatch cleavage (CCM) assay, we were the first to identify missense mutations within the TßR-II serine-threonine kinase domain in human cancer cell lines [Garrigue-Antar, 1995 #717]. These findings raised two important questions: (1) Do such structural alterations of the TßR genes also occur in primary tumors (particularly

breast cancers) *in vivo*? and, if so (2) How do mutations in the TßR genes affect receptor function?

a. GENOMIC ANALYSIS

Selection of breast cancers for genomic analysis. In collaboration with our breast pathologist, Dr. Darryl Carter, we selected a series of 36 primary stage I and -II breast carcinoma specimens for which both frozen and paraffin-embedded material is available. In 12 of these cases, we also had lymph node metastatic lesions available for analysis. We have completed the molecular structural analysis of the TßR-I and -II genes in this series. The final results are presented here:

Tissue specimens and nucleic acid extraction: Breast carcinoma specimens were provided by the Program for Critical Technologies in Breast Oncology at Yale after hisopathological review by one of us (D.C.). Genomic DNA was extracted from tumor and normal tissues as previously described (18). Isolating genomic DNA from a single 5 µm microdissected paraffin-embedded tumor section using InstaGene matrix (Bio-Rad, Hercules, CA) typically yielded 200 µl of DNA template solution. Total cellular RNA was extracted from three or more 50 µm serial thick frozen sections using TriZOL® reagent (GIBCO-BRL).

Genotyping of TGFß signaling intermediates: The TßR-II gene was analyzed by chemical mismatch cleavage as previously described (19), or by conventional PCR-SSCP (For primers used to amplify TßR-II exons. The TßR-I gene was analyzed by "cold" PCR-SSCP. In this case, each 20-µl PCR reaction contained 500 nM of unlabeled primers. Following an initial 3 minute denaturation at 95°C, PCR was performed for 35 cycles of 95°C for 30 seconds, 55°C for 40 seconds, 72° for 30 seconds followed by a 5 min final extension at 72°C. For PCR amplification of the GC-rich exon 1 we used the Advantage-GC genomic polymerase mix (Clontech Palo Alto, CA) according to the instructions supplied by the manufacturer. The 9 exons of the TßR-I gene were amplified using the following flanking intronic forward and reverse primers: Exon 1: 5'-gaggcgaggtttgctggggtgaggca-3' and 5'-catgtttgagaaagagcaggagcgag-3'; exon2: 5'-ctacacaatctttctctttttcc-3' and 5'-gtttttcttgtagtatctagg-3'; exon 3: 5'-gtttatttcactcgaggcc-3' and 5'-ggagaaacaattatgttac-3'; exon 4: 5'-gattgtgttgagtactattta-3' and 5'-ggaaaagcaaatgttacagac-3'; exon 5: 5'-gcccaaccgaaatgttaattc-3' and 5'-ggtagaactgcttatagaat-3'; exon 6: 5'-gcagtcatgtttaatttttgattc-3' and 5'-gaacgcgtattaaatatagttg-3'; exon 7: 5'-tgtctgaaaggaggttcatcc-3' and 5'-gaacaacttctgctcatgacg-3'; exon 8: 5'-gccttgcattagctgaataaat-3' and 5'-gcttactaagcagaagcag-3'; exon 9: 5'-ggaaaatggtgcatgcatta-3' and 5'-gagttcaggcaaagctgtag-3'. For SSCP analysis, 5 µl aliquots of amplified PCR product were mixed with 15 µl loading buffer (12.5 µl 10x TBE buffer, 2 µl of 15% Ficoll, 0.1% bromophenol blue & xylene cyanol, 0.5 µl methyl mercury hydroxide), denatured by heating at 80°C for 3 minutes, and quenched on ice. The single stranded DNA fragments were then resolved using precast 20% TBE acrylamide gels on a Novex Xcell II Thermoflow apparatus (Novex, San Diego, CA) with the gel temperature precisely maintained at 10°C throughout the run. Bands were visualized by staining the gel in a 1:10,000 dilution of SYBR™ Green II (Molecular Probes, Inc., Eugene, OR) for 20-30 minutes and using an Eagle Eye charged coupled device camera equipped with a SYBR™ Green band pass filter (Stratagene) for photographic documentation.

Suspect bands were excised from the gels with a razor blade and reamplified. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA), and subjected to DNA sequencing using a thermocycling sequencing kit (Epicentre® Technologies, Madison, WI) with either a forward or reverse primer end-labelled with [γ-^{32}P]-ATP. Reaction products were denatured at 70°C for 3 minutes, resolved on 7% (w/v) denaturing polyacrylamide gels at 50°C and visualized by exposing dried gels to X-ray film overnight at 20°C. The presence of any sequence alteration was always confirmed by repeated PCR-SSCP and DNA sequencing using an independent aliquot of tumor-derived genomic DNA as template. Whether any mutations were somatic in nature or present in the germline was determined by analyzing genomic DNA isolated from non-cancer tissue of the same patient.

TßR Gene Expression in Primary Breast Cancer: In order to test the hypothesis that breast carcinomas *in vivo* are refractory to TGFß, we analyzed the molecular characteristics of the two cell surface receptor genes, TßR-I and -II. TßR expression was determined using a reverse transcription-PCR assay in 14 frozen surgical breast cancer specimens from which we were able to extract good quality RNA. Each of these samples expressed both TßR-I and -II mRNA transcripts (data not shown). This is in contrast to our previous studies in esophageal- and small cell lung cancers, in which loss of TßR-II mRNA was found in 25% and 100% of cases, respectively .

Structural Analysis of the TßR-II Gene in Primary Breast Cancer: The entire open reading frame of TßR-II was screened for the presence of mutations by chemical mismatch cleavage or by PCR-SSCP followed by

DNA sequencing. No DNA sequence alterations were encountered in a total of 30 cases examined. Thus, the TßR-II gene is normally expressed in primary human breast cancer, and mutations of this gene are probably rare. This result is perhaps not surprising in light of previous studies of other cancer types: Missense and/or nonsense mutations in the TßR-II gene have only been found sporadically in colorectal- and head-&-neck cancers and in cutaneous T-cell lymphomas. The only exceptions are tumors that are associated with DNA mismatch repair deficiencies which frequently display TßR-II nonsense mutations.

Structural Analysis of the TßR-I Gene in Primary Breast Cancer: In order to determine whether mutations in the TßR-I gene might be found in human breast cancer, we screened each of the 9 exons of the TßR-I gene by PCR-SSCP in 31 primary breast carcinoma specimens and in 12 associated lymph node metastases. Areas of tumor tissue were isolated from paraffin sections by microdissection and the remaining surrounding breast tissue was used to extract germline genomic DNA. Individual exons were amplified by PCR and the products screened for the presence of novel single-strand conformation polymorphisms. Suspect bands were re-amplified and subjected to direct DNA sequencing. Individual sequence abnormalities were confirmed by repeating the entire procedure using a second aliquot of genomic DNA.

Our most important finding was a C to A transversion at nucleotide 1160 in exon 7 of TßR-I, which predicts for a serine to tyrosine substitution at codon 387. This was the only mutation encountered in the entire series and was present in 7 of the 43 specimens (16%, 95% CI: 7-31%). This is the first report of a mutation in the TßR-I gene in any type of human malignancy. Moreover, this mutation may be specifically associated with breast cancer as we have not found it in any cervical carcinomas nor in head-&-neck cancer cell lines (V.F. Vellucci and M. Reiss, unpublished data). In addition, Pasche et al. recently reported the absence of TßR-I mutations in acute myeloid leukemias.

Our second major finding is the highly significant association between the S387Y mutant and axillary lymph node metastases : While we encountered this mutation in only 2 of 31 (6%, 95% CI: 1-21) primary breast cancer specimens, it was present in 5 of the 12 (41%, 95% CI: 15-72) lymph node metastases (Fisher's Exact Test, p=0.012). The dramatically increased frequency of this mutation in lymph node metastases indicates that inactivation of the TGFß pathway may represent a late event in breast cancer progression. The fact that most breast carcinoma cell lines are refractory to TGFß is also consistent with this idea, as most of these cell lines were initially derived from metastatic cells isolated from malignant pleural effusions or ascites. Moreover, in animal models of skin carcinogenesis, TGFß resistant tumor cell clones also do not emerge until the tumors have become highly aggressive and metastatic. In contrast, in colorectal cancers associated with DNA mismatch repair deficiencies, the acquisition of TßR-II gene mutations appears to coincide with the transition from pre-invasive adenoma to invasive carcinomas. Thus, the stage of tumor development at which the TGFß signaling pathway becomes inactivated appears may vary depending on the tumor type and on the underlying molecular genetic events that drive the carcinogenetic process.

As the S387Y mutation was not detected in germline DNA of the same individuals, we can practically exclude the possibility that this sequence alteration represents a normal polymorphism. On the other hand, besides the mutant band, a wild type band could be detected in each of the tumor specimens. Although these findings suggest that the tumors may have retained a wild type allele, it is impossible to exclude the possibility that this wild type band was the result of the almost inevitable contamination of the specimens with at least some normal cells. However, even loss of function of one of the two TßR-I alleles may be sufficient to confer a significant a selective advantage. Such a dosage effect occurs, for example, in transgenic animals that express a dominant-negative TßR-II gene in conjunction with two endogenous wild type alleles, and in knock-out mice that carry only a single TGFß1 gene allele. In both of these situations, the animals are significantly more susceptible to tumor formation.

Besides the S387Y somatic missense mutation, we also detected a variant allele of the TßR-I gene with an in-frame deletion of 3 of 9 repeating GGC trinucleotides within exon 1. Thirteen of 24 evaluable cases with BC were heterozygous carriers of this del$(GGC)_3$ TßR-I variant (54%, 95% C.I. 33-74%). This deletion results in the loss of 3 of the 9 alanine residues that constitute the hydrophobic core of the putative TßR-I signal. Comparative hydrophobicity plots of wild type and the del $(GGC)_3$ TßR-I variant clearly show that the deletion shortens the hydrophobic core of the signal peptide . These findings suggested that this deletion may well have functional consequences for the receptor protein, particularly its ability to be targeted to the cell membrane.

In order to determine whether there might be an association between the carrier state of the del$(GGC)_3$

TßR-I variant and the development of breast cancer, we determined the frequency of the del$(GGC)_3$ allele in a cohort of germline DNA samples from 43 independently and randomly selected individuals. Only one of these individuals was heterozygous for the del$(GGC)_3$ variant of TßR-I (2%, 95% CI: 0-12%). This translates into a highly significant increased relative risk of developing BC in carriers over control (Fisher's Exact test: $p<0.0001$)(Relative risk: 3.18; 95% C.I.: 2.32-4.36). These findings strongly argue in favor of the hypothesis that the del$(GGC)_3$ variant of TßR-I confers an increased cancer risk, presumably by decreasing the sensitivity of normal breast epithelial cells to TGFß.

Case-control Study: In order to test the validity of these results, we have taken advantage of the recently completed Yale Environment and Breast Disease Study. In this prospective case-control study, Dr. Tongzhang Zheng has been testing the hypothesis that exposure to organochloride pesticides increases the risk of BC. Close to 400 cases and 200 control women were enrolled between January 1994 and August 1997. All cases had histologically confirmed diagnoses of primary BC (TNM stages 0-III). Standardized structured questionnaires were used to ascertain demographic factors, menstrual and reproductive history, past medical history and family history of cancer, occupation, household pesticide use, use of hair dyes, alcohol and tobacco, and dietary history. In addition, blood clots were stored frozen to be used for future studies of genetic polymorphisms. The epidemiological data that have been collected and the availability of genomic DNA from all cases and controls represented an invaluable opportunity for us to rigorously test the idea that the del$(GGC)_3$ TßR-I gene variant may represent a novel and common breast cancer susceptibility gene.

Cases (n=98) were selected from among previously ascertained subjects who participated in the Yale Environment and Breast Disease Study. All cases had histologically confirmed primary BC (stages 0-III). Age-matched controls (n=92) were selected from among the women in the same study who did not have a diagnosis of BC. Eleven cases (11%, 95% CI: 6-19%) and 14 of the controls (15%, 95% CI: 9-24%) were heterozygous carriers of the del$(GGC)_3$ TßR-I gene variant. These results indicate that there was no significant association between the del$(GGC)_3$ TßR-I gene variant carrier state and breast cancer (Fisher's Exact test, $p=0.52$).

In summary, in this initial series of primary breast cancers, we identified one particular structural alterations of the TßR-I gene that appears to be uniquely associated with breast carcinomas, and is found more frequently in axillary lymph node metastases than in primary tumors. In order to confirm these findings, Dr. Daryl Carter provided us with an additional 24 cases of axillary lymph node metastases from breast carcinoma. Tumor tissue was microdissected, and genomic DNA extracted as described above. Exon 7 of the TßR-I gene was analyzed by PCR-SSCP. In one single case, we detected and confirmed the presence of the identical S387Y mutation found in the initial series. Thus, these results further support our hypothesis that mutations of the TßR-I gene represent relatively late events in breast cancer progression.

Detection of TßR-I and -II Gene Losses by FISH: For cells to loose all responsiveness to TGFß, both alleles of any one of the signaling intermediate genes need to be inactivated. In analogy with other tumor suppressor genes, this is likely to be a two-step process involving loss of one allele and inactivation of the second allele by intragenic mutation. Allelic deletions are often identified by using PCR-based assays for the detection of polymorphic DNA sequences. This approach has several drawbacks: First, it requires the availability of paired tumor- and germline DNA samples. Secondly, such assays are informative only if the individuals are heterozygous for the marker used. Finally, and most importantly, the test will only yield a positive result if the majority of tumor cells has undergone loss of heterozygosity (LOH). Thus, PCR-based approaches will fail to detect allelic losses if they are present in only a minority of the tumor cells.

Fluorescent *in situ* hybridization (FISH) is a particularly attractive alternative method for detecting LOH because it does not require access to normal tissue from the same individual and can be used to detect changes in gene copy numbers in individual cells. Moreover, FISH has been used effectively to detect allelic losses in interphase nuclei in tissue sections or touch preparations of tumor samples.

The main purpose of this study was to determine whether the genes that encode the two TGFß receptors (TßR-I and TßR-II) undergo allelic deletions during breast cancer development and progression. We approached this question by examining interphase nuclei in breast cancer specimens by FISH. A total of 18 primary cancer specimens were examined. These included 15 invasive ductal cancers, 2 invasive lobular carcinomas, and 1 intracystic papillary cancer. Interphase nuclei were hybridized with BAC clones containing the complete genomic sequences of either TßR-I or TßR-II. Specimens were co-hybridized with centromeric probes for the corresponding chromosomes (chromosome 9 for TßR-I, chromosome 3 for TßR-II).

The results for all 18 cases of primary breast cancer have been depicted graphically in **Figure 1**. In most cases, we could identify subpopulations of nuclei in which the number of TßR-specific signals was less than 2. However, the hybridization efficiency of locus-specific DNA probes is probably lower than that obtained with the repeat-sequence probes used to identify centromeres, because the signals are smaller and less intense than centromeric signals. In order to estimate the proportion of false-negative TßR gene signals, we examined touch preparations of 4 different normal axillary lymph nodes that had been obtained at the time of breast surgery and were processed in a manner identical to the tumor samples. The average fraction of nodal lymphocytes with <2 TßR-specific signals was 19% (95% CI: 9-29) for TßR-I and 21% (95% CI: 3-38) for TßR-II. Using the upper boundaries of the 95% confidence intervals as threshold values (29% for TßR-I and 38% for TßR-II), we concluded that tumor cell subpopulations with bona fide TßR-I deletions were present in 2 of 6 (33%), and TßR-II deletions in 6 of 10 (60%) touch preparations . In all cases, approximately half of the losses involved both copies of the TßR-I or -II gene.

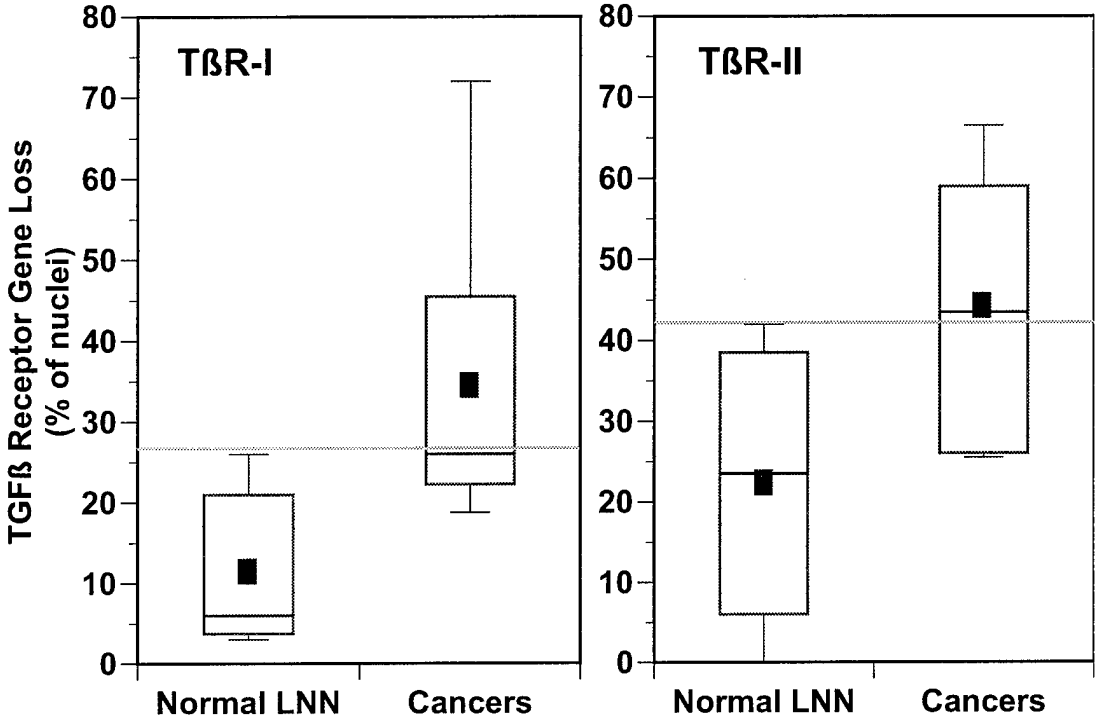

Figure 1. TGFß Receptor Gene Losses in Primary Breast Carcinomas

Our results indicate that approximately 50% of the primary invasive carcinoma specimens contained subpopulations of cells that had undergone allelic losses of either the TßR-I or the TßR-II gene. These findings raised the question at which stage of tumor development these losses had occurred. To answer this question, we will have to examine a series of cases that span the spectrum of pre-invasive to metastatic breast cancer. However, our data provide some preliminary insight. For example, in the single case of non-invasive intracystic papillary carcinoma (6T), we found no evidence of significant allelic loss of either of the two receptor genes. We also examined a single metastatic lesion. This chest wall recurrence demonstrated extensive aneuploidy of both chromosomes 3 and 9 with increasing allelic loss of the TBR genes with increasing chromosome copy number. However, in spite of the high frequency of chromosomal gains and losses, losses of both TßR genes in this case were of the same order of magnitude as those seen in the primary tumors. Thus, these findings suggest that losses of genes that encode TGFß signaling intermediates may occur progressively as breast cancers evolve from pre-invasive to invasive to metastatic lesion.

b. IMMUNOSHISTOCHEMICAL STUDIES:

Generation of anti-phospho-Smad2 antibody: FISH and PCR-SSCP are labor-intensive and technically challenging approaches to identifying lesions in the TGFß signaling pathway that do not lend themselves well to the analysis of large numbers of tumor specimens. Two key features of TGFß signaling can be exploited to gain a better understanding of TGFß signaling in tumor sections. These include the phosphorylation of Smad2 and –3 by activated TGFß receptors, and the nuclear localization of these phosphorylated Smads in transcription complexes. In order to test the validity of this approach, we have developed activation state-specific antibodies directed against

Smad2 and -3. We postulate that loss of expression of Smad4 and/or phosphorylation of Smad2 and -3 accurately predicts the underlying molecular mechanism of TGFß-resistance.

Activation-state specific anti-Smad antibodies: In order to be able to distinguish between the activated (phosphorylated) and inactive forms of Smad2 and -3, we raised polyclonal rabbit antibodies against synthetic peptides comprising the C-terminal 13 amino acids of Smad2 or Smad3, in which two phosphoserine residues were incorporated at the extreme C-terminus (KMGSPSVRCSS^{P}MS^{P} and KMGSPSIRCSS^{P}VS^{P}, respectively), coupled to keyhole limpet hemocyanin (KLH) as carrier protein. The antisera were affinity-purified by negative selection using a KLH-agarose column, followed by chromatography using an Affigel-10 (BioRad) matrix to which unphosphorylated Smad2 and Smad3 peptides had been coupled. The final purification step consisted of a positive selection using the appropriate phosporylated Smad2 (Smad2P) or Smad3 (Smad3P) peptide coupled to Affigel-10 matrix. The antibody was eluted using 3M sodium thiocyanate, immediately neutralized using 100 mM Tris and dialyzed against phosphate buffered saline (PBS) for 48 hours. The specificity and sensitivity of the anti-Smad2P and anti-Smad3P antibodies were confirmed by ELISAs against .BSA conjugates of phosphorylated versus unphosphorylated peptide.

Detection of Smad expression by Western blotting: The sensitivity and specificity of the Smad and phospho-Smad antibodies were tested by Western blotting of extracts of breast carcinoma cells treated with TGFß or vehicle only. Cells were grown to confluence in 100-mm dishes and treated with 100 pM TGFß for 1 hour. Cells were then lysed *in situ* in buffer composed of 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 1% (v/v) TritonX-100, 1 mM phenylmethyl sulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, MO), 20 µg/mL of aprotinin (Sigma), and 25 µg/mL of leupeptin for 30 min at 4°C. After clarification of the lysates by centrifugation, protein extracts were resolved by SDS-PAGE and transferred to nitrocellulose paper in a buffer composed of 25 mM Tris-HCl (pH 8.0), 192 mM glycine, 20% (v/v) methanol, using an Owl Scientific electroblotting apparatus (USA Scientific Plastics, Ocala, FL). Duplicate filters were then incubated for 30 min. at 20°C in blocking buffer containing PBS supplemented with 5% (w/v) Carnation dry milk and 0.1% (v/v) Tween-20, followed by incubation for 12 to 16 hr at 4°C in PBS containing 1 µg/mL of anti-Smad peptide antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or 1 µg/mL of anti-Smad antibody that had been pre-incubated with a 10-fold molar excess of the cognate peptide. Smad2 and –3 were detected using a goat polyclonal dual specificity anti-Smad2/3 antibody (N-19, Santa Cruz). Smad4 was detected using a mouse monoclonal anti-Smad4 antibody (B-8, Santa Cruz). Smad2P and Smad3P were detected using our own rabbit anti-Smad2P and –3P antibodies (see above). Blots were developed using a 1:2000 dilution of horseradish peroxidase-tagged goat anti-rabbit or –mouse IgG (Calbiochem, San Diego, CA) and the bands visualized using DuPont NEN Chemiluminescence Reagent as recommended by the manufacturer.

We analyzed Smad4 and Smad2P expression in a panel of human breast carcinoma cell lines as well as the non-neoplastic mouse mammary epithelial line, HC-11. Smad4 protein was expressed in HC-11 cells, as well as in 8 of the 10 BC lines. However, 2 of the BC lines (ZR75-1 and MDA-MB-468) failed to express any Smad4 protein. Moreover, TGFß treatment had no effect on Smad4 expression. In contrast, each of the BC lines expressed equal levels of Smad2 (not shown). We then examined the effects of TGFß treatment on Smad2 phosphorylation. As shown in Figure 4B, while phospho-Smad2 was not detectable in the absence of TGFß, it became easily detectable within 1 h of the addition of 100 pM TGFß1 to HC-11 cells. Phosphorylation of Smad2 was induced by as little as 10 pM of TGFß1, and detectable within 15 min. (data not shown). Besides in HC-11 cells, TGFß treatment induced phosphorylation of Smad2 in 9 of 10 BC cell lines, indicating that these cell lines expressed functionally intact TGFß receptors. In contrast, no phosphorylation of Smad2 was observed in T47D cells, which are known to lack TßR-II expression. Thus, the absence of phospho-Smad2 can be used as a surrogate marker of a TGFß receptor defect.

Tissue microarray studies: In order to assess the status of TGFß signaling in a large cohort of primary human breast carcinoma specimens, we analyzed tissue microarrays that contained a total of 135 cases of primary breast carcinomas. Consecutive sections of the microarrays were stained with hematoxylin-eosin, anti-Smad2 (Santa Cruz), anti-phospho-Smad2, and anti-Smad4 (Santa Cruz). The results are summarized in **Table 1**.

We encountered three types of cases: The majority (88%) expressed both Smad2 and Smad4, as well as activated Smad2P. These findings indicate that biologically active TGFß was present in these tumors, and that the TGFß receptors were actively signaling. In 10 cases (14% of total), Smad2 was expressed, but Smad2P was not detectable. These findings indicate the presence of defective receptors in these cases; we are currently examining these tumors for structural alterations in the TßR-I or –II genes. In addition, 6 of the cases failed to express Smad4 protein. Interestingly, 2 cases that failed to express Smad2P also failed to express Smad4. Recent studies

by Hruban et al have shown that failure to detect Smad4 by immunohistochemistry strongly predicts for the presence of mutation or deletion of the Smad4 gene. Thus, these 2 breast carcinomas appear to have a dual defect in the TGFß pathway that involves both loss of Smad4 and a TßR defect. Similar dual defects have recently been described in several carcinoma cell lines derived from pancreatic-, colorectal-, and head-&-neck cancers.

Table 1. Expression of Smads in human primary breast cancers

	Positive	Negative	Total
Smad2	116 (100%)	0	116
Smad2P	106 (86%)	10 (14%)	116
Smad4	110 (95%)	6 (5%)	116

Plans for the coming year include extension of these studies on tissue microarrays in two directions: 1. First, we intend to analyze much larger cohorts of primary breast cancers to reliably determine the frequency of TGFß receptor defects and Smad4 inactivation, and to determine whether inactivation of TGFß signaling has prognostic significance. 2. Secondly, we plan to analyze a large cohort of cases for which we have both primary tumor tissue and axillary lymph node metastases. This will allow us to test the prediction that inactivation of the TGFß signaling pathway is particularly associated with the metastatic phenotype.

Task 2. Determination of the functional consequences of TGFß-receptor mutations

In order to test whether the serine to tyrosine substitution at position 387 found in primary and metastatic breast cancer specimens disrupts receptor function, we introduced this mutation into a full-length wild type TßR-I cDNA. We studied the effects of the mutation on receptor function in transient transfection assays using the TßR-I-deficient R-1B (L17) mink lung epithelial cell line. Expression of wild type TßR-I in R-1B (L17) cells resulted in an approximately 50% reduction in cyclin A promoter activity compared to cells transfected with an inert control vector. In contrast, pCAL2 activity was repressed by less than 30% in cells transfected with the S387Y receptor mutant. In cells transfected with wild type TßR-I, pSBE4-dependent luciferase activity was increased approximately 15-fold over controls, while the increase observed in cells transfected with the receptor mutant was only approximately 10-fold. The S387Y mutation appears to induce a shift in the TGFß dose-response relationship: wild type TßR-I expressing cells responded maximally to 50 pM TGFß, whereas S387Y expressing cells required at least 100 pM TGFß for maximal response. Repression of the cyclin A promoter activity (pCAL2) correlates extremely well with the ability of cells to respond to TGFß-mediated cell cycle arrest, and activation of the Smad DNA-binding element (SBE) in pSBE4 reflects TGFß-induced gene transcription. To rule out that the observed differences in reporter gene activity were due to variations in levels of expression of wild type and mutant TßR-Is in transfected cells, cell lysates were subjected to Western immunoblotting using anti-HA monoclonal antibody. Discreet 55 kDa bands of equal intensity corresponding to the TßR-I receptor were detected in extracts from both wild type- and mutant TßR-I-transfected cells. Thus, the S387Y mutation did not affect receptor protein expression. In summary, cells expressing the S387Y mutant was significantly less sensitive to the effects of TGFß on cell cycle regulation as well as transcriptional responses than cells expressing the wild type receptor.

The exact mechanism whereby the S387Y mutation diminishes TGFß signaling remains to be determined. According to the canonical domain subdivisions found in all protein kinases, the serine residue at position 387 in TßR-I is located in the linker region between subdomains VIII and IX which typically form the peptide recognition domain of protein kinases. The structure of subdomains VIII and IX are highly conserved among the family of type I TGFß-, activin- and bone morphogenic protein (BMP) receptor serine-threonine kinases. The fact that these receptors share highly homologous substrates (Smads) further suggests that this region participates in substrate recognition. Alternatively, it may well affect the homodimerization of TßR-I molecules, or perhaps the interactions between TßR-I and –II molecules when they form heterotetrameric complexes during receptor activation.

The primary substrate of TßR-I, Smad2, is phosphorylated on two serine residues located within the consensus sequence RCSS(465)MS(467) at the C-terminus of the protein. Although the C-terminal tail of Smad2

is not absolutely required for its physical interaction with TßR-I, structure-function studies indicate that it clearly plays a complimentary role in enzyme-substrate recognition and partly determines specificity between TGFß and BMP signaling. Comparison between the crystal structures of activated protein kinase A in complex with an inhibitory peptide and that of the TßR-I kinase indicates that the S387Y residue does not fall precisely within the canonical substrate binding site as defined in the protein kinase A-inhibitory peptide structure. However, this does not exclude the possibility that the substitution of a tyrosine with its larger side chain for the serine at position 387 in TßR-I interferes with productive substrate recognition, particularly as the interface between a Smad and TßR-I is probably much larger than the protein kinase A-inhibitory peptide interface. Furthermore, based on the Chou and Fassman algorithm, one would predict that the S387Y mutation alters the secondary structure of the TßR-I kinase by introducing two ß-sheets flanking the loop that connects the E and F α-helices of the catalytic core. It is worth noting that two other TGFß type I receptors, TSR-1 and TskL7, contain different polar residues at position 387 (threonine and glutamine, respectively). Interestingly, neither of these two receptors is able to elicit the same cellular responses as TßR-I, perhaps because they are unable to interact with Smad2 or –3.

Finally, the functional importance of this region is also illustrated by the fact that several syndromes have been associated with mutations within subdomains VIII or IX in other protein kinases. For example, two different arginine-to-tryptophan and methionine-to-arginine mutations in the TSR-1 gene have been described in hereditary haemorrhagic telangiectasia type 2. Moreover, amino acid substitutions at highly conserved glutamate and aspartate residues in the catalytic subunit of phosphorylase B kinase result in loss of enzyme activity, glycogenosis and liver cirrhosis. In addition, Wang et al. recently described a case of head-&-neck cancer with a tyrosine-to-cysteine mutation within subdomain IX of the TßR-II serine-threonine kinase. Although the effects of this mutation on receptor function were not reported in this case, it is likely that it affects enzyme activity as well.

In summary, we have identified a single missense mutation of the TßR-I gene that occurs with relatively high frequency in invasive ductal breast cancer and that has a significant negative impact on receptor signaling. This is the first reported missense mutation in this gene reported in any human malignant neoplasm and provides further support for the idea that inactivation of the TGFß signaling pathway can play an important role in human carcinogenesis. Furthermore, the high frequency of the S387Y mutation in lymph node metastases suggests that inactivation of this signaling pathway may be particularly associated with the metastatic phenotype.

Task 3. To determine the potential clinical significance of genetic alterations of the TßR genes in breast cancer

We are addressing the clinical significance of TGFß signaling in two different ways:

First, we intend to use tissue microarrays to analyze a large cohort of primary breast cancers to reliably determine the frequency of TGFß receptor defects and Smad4 inactivation, and to determine whether inactivation of TGFß signaling has prognostic significance. Preliminary studies have shown that archival material from as far back as 1950 can be reliably stained with the Smad2, Smad2P and Smad4 antibodies. Thus, we will be able to generate tissue microarrays from tumor samples for which long-term (>20 years) follow-up information is available through the Connecticut Tumor Registry.

Secondly, we had proposed to test the hypothesis that the actions of the anti-estrogen, tamoxifen, are mediated by the induction of biologically active TGFß. If this assumption is correct, we predicted that defects in TGFß signaling might explain the cases of tamoxifen resistance among estrogen receptor-positive breast cancers. We have begun to address these questions in a preliminary study conducted in collaboration with Drs. Lorna Marson and William Miller of the University of Edinburgh. These investigators have compiled a series of breast biopsies taken from patients prior to and 6 months following the start of tamoxifen therapy. We have examined the TGFß signaling pathway using our immunohistochemical approach in 10 paired specimen sets obtained from Edinburgh. The results are summarized in **Table 2**.

The results of this pilot study indicate that each of the 9 evaluable cases expressed Smad2P. However, in the 4 non-responders, Smad2P immunostaining was not increased in the post-treatment samples, whereas Smad2P expression was clearly increased in 2 of the 4 evaluable responders. Although this small feasibility does not allow us to draw any conclusions, we intend to complete an analysis of the entire cohort of 100 paired specimens from Edinburgh.

Table 2. Smad2 activation in breast cancers as a function of tamoxifen therapy

#	Histology	Smad2P expression	Response?
1A	No cancer No normal ducts	N/A N/A	
1B	Normal ducts DCIS	1-2+ 1-2+	Yes
2A	Invasive carcinoma	1+	
2B	Normal ducts DCIS Invasive carcinoma	0-1+ 0-1+ 1+	No
3A	Invasive carcinoma (mucinous)	1+	
3B	Skin Normal ducts Invasive carcinoma	2+ 1-2+ 1-2+	Yes
4A	DCIS Invasive carcinoma	1-2+ 1-2+	
4B	Normal ducts ADH Tubular carcinoma	0-1+ 0-1+ 0	Yes
5A	Normal ducts Invasive carcinoma	1+ 1-2+	
5B	Normal ducts No carcinoma seen	0	Yes
6A	Tubular carcinoma	1+	
6B	Tubular carcinoma	1-2+	Yes
7A	Normal ducts DCIS Invasive carcinoma	0 0-1+ 1-2+	
7B	Normal ducts ADH DCIS Invasive carcinoma	0 0 0 1+	No
8A	Normal ducts Invasive carcinoma	1-2+ 1-2+	
8B	Normal ducts Invasive carcinoma	0 0	No
9A	Normal ducts Invasive carcinoma	1-2+ 1-2+	
9B	Invasive carcinoma	0	No

Key research accomplishments:

- Detection of allelic losses of TGFß receptor genes in primary human breast cancer
- Identification of the first missense mutations in the TGFß type receptor gene in human (breast) cancer
- Identification of TßR receptor defects and Smad4 losses in primary human breast cancer tissue microarrays

Reportable outcomes:

- Generation of phospho-Smad2-specific antibody
- **Reiss, M.** and Barcellos-Hoff, M.H. Transforming Growth Factor-ß in breast cancer-a working hypothesis-Breast Cancer Res. & Treatment. 1997. 45:81-95.
- Chen, T., Carter, D., Garrigue-Antar, L., and **Reiss, M.** Transforming Growth Factor-ß type I receptor kinase mutant associated with metastatic breast cancer. Cancer Res. 1998. 58:4805-4810.

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Advances in Brief

Transforming Growth Factor β Type I Receptor Kinase Mutant Associated with Metastatic Breast Cancer[1]

Taiping Chen, Darryl Carter, Laure Garrigue-Antar,[2] and Michael Reiss[3]

Departments of Medicine [T. C., L. G-A., M. R.] and Pathology [D. C.], Yale Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06520-8032

Abstract

Malignant breast carcinoma cell lines are frequently refractory to transforming growth factor β (TGF-β)-mediated cell cycle arrest. To identify molecular mechanisms of TGF-β resistance, we have conducted a comprehensive structural analysis of the TGF-β receptor types I (*TβR-I*) and II (*TβR-II*) genes in primary human breast carcinomas and associated axillary lymph node metastases. No evidence for loss of expression (n = 14) or structural alterations of the *TβR-II* gene (n = 30) were identified. However, 2 of 31 primary carcinomas and 5 of 12 lymph node metastases carried a C to A transversion mutation resulting in a serine to tyrosine substitution at codon 387 (S387Y) of the *TβR-I* receptor gene. This *TβR-I* mutant has a diminished ability to mediate TGF-β-dependent effects on gene expression as compared with wild-type *TβR-I*. S387Y is the first reported mutation in the *TβR-I* gene in human cancer that was primarily associated with lymph node metastases in the present series.

Introduction

TGF-β[4] is a M_r 25,000 dimeric polypeptide that is the most potent known inhibitor of normal human mammary epithelial cell replication *in vitro* (1). *In vivo*, TGF-β seems to regulate the normal development of ductal and lobular epithelium in the mammary gland (2, 3). Moreover, in the adult mammary gland, TGF-β probably mediates the massive cell death and restructuring that takes place in the mammary gland during postlactational involution (4).

Besides these physiological functions, there is considerable evidence that TGF-β plays an important role in mammary carcinogenesis (reviewed in Ref. 5). First of all, TGF-β is able to protect against mammary tumor formation *in vivo*. For example, transgenic mice that produce a constitutively active form of TGF-β1 are relatively resistant to carcinogen-induced mammary tumor formation (6), Conversely, heterozygous TGF-β1 knockout mice that express lower than normal levels of TGF-β1 have an increased propensity for tumor development (7). The same holds true for mice that express a dominant-negative *TβR-II* mutant gene or that have a targeted deletion of the *TβR-II* gene (8, 9). Thus, either a relative lack of TGF-β or inactivation of the TGF-β signaling pathway results in loss of tumor suppression and promotes carcinogenesis. Secondly, many mammary carcinomas seem to be composed of TGF-β-insensitive cells. Thus, virally transformed tumorigenic mammary epithelial cell lines as well as most of the cell lines derived from invasive human breast carcinomas are resistant to the antiproliferative effects of TGF-β *in vitro* and do not respond to treatment with TGF-β *in vivo* (5). These observations have raised the question of what is the molecular basis for TGF-β resistance in breast cancer.

The TGF-β signal is transduced by a pair of transmembrane serine-threonine kinase receptors (10). TGF-β binds primarily to TβR-II receptor homodimers, which then form heterotetrameric complexes with two TβR-I molecules. As a consequence, the TβR-II kinase phosphorylates TβR-I thereby activating its serine-threonine kinase. In response to TGF-β binding, the two cytosolic proteins, Smad2 and Smad3, become transiently associated with and phosphorylated by the TβR-I kinase. Following their activation, Smad2 and -3 form heteromeric complexes with a third homologue, Smad4. These complexes are translocated to the nucleus, bind to DNA in a sequence-specific manner, and regulate gene transcription (10). The resulting repression of cyclins and induction of cyclin-dependent kinases and cdc25A phosphatase lead to G_1 phase cell cycle arrest.

A number of breast carcinoma cell lines have been described that fail to express either the *TβR-II* or the *Smad4* gene and are refractory to TGF-β (11–14). In two of these lines, intragenic mutations of the *Smad4* gene were noted in conjunction with loss of the second allele (13, 14). On the basis of these observations, one would predict that the TGF-β signaling pathway would be disrupted in primary breast carcinomas *in vivo* as well. However, Riggins *et al.* (15) failed to identify any structural alterations of the *Smad1*, *-3*, *-5*, or *-6* genes in over 20 breast cancer cell lines. Moreover, other investigators have found the *Smad2* and *-4* genes to be intact in substantial numbers of primary breast carcinoma specimens (16, 17). Thus, we are faced with the apparent paradox that most breast carcinoma cell lines are refractory to TGF-β *in vitro*, whereas the inactivation of the *Smad* genes in breast carcinoma specimens seems to occur quite infrequently. These findings suggest that the *TβR* genes may be the primary targets for genetic inactivation in this disease.

To address this possibility, we have investigated the *TβR-I* and *-II* genes in a panel of primary breast carcinomas and associated axillary lymph node metastases. We have identified a particular somatic missense mutation within the catalytic core of the TβR-I serine-threonine kinase that disrupts the signaling function of the receptor. This is the first inactivating mutation of the *TβR-I* gene described in human cancer. Moreover, our findings indicate that inactivation of the TGF-β signaling pathway in sporadic breast carcinoma is probably a relatively late event because the mutation was found predominantly in metastatic lesions. This may partly explain the fact that previous studies have failed to uncover molecular evidence for TGF-β pathway inactivation because they have focused exclusively on primary tumor specimens.

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[2] Present address: Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom.

[3] To whom requests for reprints should be addressed, at Section of Medical Oncology, Department of Internal Medicine and Yale Cancer Center, NS291, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8032. Phone: (203) 785-6221; Fax: (203) 785-7531; E-mail: michael.reiss@yale.edu.

[4] The abbreviations used are: TGF-β, transforming growth factor β; TβR-I, type I TGF-β receptor; TβR-II, type II TGF-β receptor; SSCP, single-strand conformation polymorphism; TBE, 89 mM Tris, 89 mM borate, 50 mM EDTA (pH 8.0); BMP, bone morphogenic protein; CMV, cytomegalovirus.

Materials and Methods

Tissue Specimens and Nucleic Acid Extraction. Breast carcinoma specimens were provided by the Program for Critical Technologies in Breast Oncology at Yale after hisopathological review by one of us (D. C.). Genomic DNA was extracted from tumor and normal tissues as described previously (18). Isolating genomic DNA from a single 5-μm microdissected paraffin-embedded tumor section using InstaGene matrix (Bio-Rad, Hercules, CA) typically yielded 200 μl of DNA template solution. Total cellular RNA was extracted from three or more 50-μm serial thick frozen sections using TriZOL reagent (Life Technologies).

Genotyping of TGF-β Signaling Intermediates. The *TβR-II* gene was analyzed by chemical mismatch cleavage as described previously (19) or by conventional PCR-SSCP. (For primers used to amplify TβR-II exons, see Ref. 20.) The *TβR-I* gene was analyzed by "cold" PCR-SSCP (21). In this case, each 20-μl PCR contained 500 nM of unlabeled primers. After an initial 3-min denaturation at 95°C, PCR was performed for 35 cycles of 95°C for 30 s, 55°C for 40 s, 72° for 30 s followed by a 5-min final extension at 72°C. For PCR amplification of the GC-rich exon 1 we used the Advantage-GC genomic polymerase mix (Clontech, Palo Alto, CA) according to the instructions supplied by the manufacturer. The 9 exons of the *TβR-I* gene were amplified using the following flanking intronic forward and reverse primers: (*a*) exon 1: 5′-gaggcgaggtttgctggggtgaggca-3′ and 5′-catgtttgagaaagagcaggagcgag-3′; (*b*) exon 2: 5′-ctacacaatctttctctttttcc-3′ and 5′-gtttttcttgtagtatctagg-3′; (*c*) exon 3: 5′-gtttatttcactcgaggcc-3′ and 5′-ggagaaacaattatgttac-3′; (*d*) exon 4: 5′-gattgtgttgagtactattta-3′ and 5′-ggaaaagcaaatgttacagac-3′; (*e*) exon 5: 5′-gcccaaccgaaatgttaattc-3′ and 5′-ggtagaactgcttatagaat-3′; (*f*) exon 6: 5′-gcagtcatgtttaatttttgattc-3′ and 5′-gaacgcgtattaaatatagttg-3′; (*g*) exon 7: 5′-tgtctgaaaggaggttcatcc-3′ and 5′-gaacaacttctgctcatgacg-3′; (*h*) exon 8: 5′-gccttgcattagctgaataaat-3′ and 5′-gcttactaagcagaagcag-3′; and (*i*) exon 9: 5′-ggaaaatggtgcatgcatta-3′ and 5′-gagttcaggcaaagctgtag-3′. For SSCP analysis, 5-μl aliquots of amplified PCR product were mixed with 15 μl of loading buffer (12.5 μl of 10× TBE buffer, 2 μl of 15% Ficoll, 0.1% bromphenol blue and xylene cyanol, and 0.5 μl methyl mercury hydroxide), denatured by heating at 80°C for 3 min, and quenched on ice. The single-stranded DNA fragments were then resolved using precast 20% TBE acrylamide gels on a Novex Xcell II Thermoflow apparatus (Novex, San Diego, CA) with the gel temperature maintained precisely at 10°C throughout the run. Bands were visualized by staining the gel in a 1:10,000 dilution of SYBR Green II (Molecular Probes, Inc., Eugene, OR) for 20–30 min and using an Eagle Eye charged coupled device camera equipped with a SYBR Green band pass filter (Stratagene) for photographic documentation.

Suspect bands were excised from the gels with a razor blade and reamplified. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA), and subjected to DNA sequencing using a thermocycling sequencing kit (Epicentre Technologies, Madison, WI) with either a forward or reverse primer end-labeled with [γ-^{32}P]-ATP. Reaction products were denatured at 70°C for 3 min, resolved on 7% (w/v) denaturing polyacrylamide gels at 50°C, and visualized by exposing dried gels to X-ray film overnight at 20°C. The presence of any sequence alteration was always confirmed by repeated PCR-SSCP and DNA sequencing using an independent aliquot of tumor-derived genomic DNA as a template. Whether any mutations were somatic in nature or present in the germline was determined by analyzing genomic DNA isolated from noncancer tissue of the same patient.

Vectors Used for Transfection. The pHA-1 mammalian expression vector was constructed by subcloning the full-length human *TβR-I* (ALK-5; Ref. 22) into the expression vector, pCDNA3 (Stratagene), thereby placing it under the transcriptional control of a CMV promoter. To facilitate the detection and quantitation of transfected receptor, the influenza virus HA epitope tag YPYDVPDYA was introduced at the COOH-terminus of the protein (23). The C to A transversion in codon 387 was introduced into the wild-type *TβR-I* sequence by site-directed mutagenesis as described previously (24).

Reporter Gene Assays. The signaling function of the mutant *TβR-I* receptor was assessed in transient transfection assays into R-1B (L17) cells, a subclone of Mv1Lu mink lung epithelial cells (a generous gift of Dr. J. Massagué, Memorial-Sloan Kettering Cancer Center, New York, NY). This cell line is convenient because it is refractory to TGF-β, fails to express detectable levels of TβR-I, and all of the responses to TGF-β can be restored by reexpressing wild-type TβR-I. Two different firefly luciferase reporter gene constructs were used to assess the different types of responses to TGF-β: (*a*) pCAL2 (a generous gift of Dr. R. Derynck, University of California, San Franscisco, CA), which contains cyclin A gene promoter (25); and (*b*) pSBE4 (a generous gift from Dr. B. Vogelstein, Johns Hopkins University, Baltimore, MD), in which four tandem repeats of a Smad4-specific DNA binding element drive the luciferase cDNA (26).

For transfections, R-1B (L17) cells were plated at 1.4×10^5 cells/well in 6-well cluster dishes in RPMI 1640 (Life Technologies) supplemented with 10% (v/v) fetal bovine serum and allowed to adhere overnight at 37°C. Transfections using up to 1 μg of TβR-I and 2 μg of reporter plasmid DNA were carried out using Lipofectin (Life Technologies) as described previously (24). To control for variations in transfection efficiency, we cotransfected a small amount (0.01 μg) of pRL-CMV, a plasmid expressing a *Renilla* luciferase reporter gene (Promega). Firefly and *Renilla* luciferase activities can be detected separately in the same cell lysates because of their different substrate specificities using the protocol provided by the manufacturer (Promega). Cell lysate was mixed with the appropriate luciferase assay reagent and photon emission was measured using a Series 20 Barthold Luminometer (Turner Designs, Sunnyvale, CA).

Receptor Expression. Cell lysates from transfected cells were prepared using buffer containing 1% [v/v] Triton X-100, 0.1% [w/v] SDS, 150 mM NaCl, 50 mM Tris (pH 7.5), 3 mM sodium azide, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin. After boiling for 10 min. in the presence of sample buffer, aliquots containing equal amounts of total protein were resolved by electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and subjected to Western immunoblotting using rabbit polyclonal antiserum directed against the HA peptide (HA.11, BAbCO, Richmond, CA). Blots were developed using horseradish peroxidase-tagged goat antimouse IgG, and the bands were visualized using DuPont NEN Chemiluminescence Reagent as recommended by the manufacturer.

Results and Discussion

To test the hypothesis that breast carcinomas *in vivo* are refractory to TGF-β, we analyzed the molecular characteristics of the two cell surface receptor genes, *TβR-I* and *TβR-II*. TβR expression was determined using a reverse transcription-PCR assay in 14 frozen surgical breast cancer specimens from which we were able to extract good quality RNA. Each of these samples expressed both *TβR-I* and *TβR-II* mRNA transcripts (data not shown). This is in contrast to our previous studies in esophageal cancers and small cell lung cancers, in which loss of *TβR-II* mRNA was found in 25 and 100% of cases, respectively (27, 28).

The entire open reading frame of *TβR-II* was screened for the presence of mutations by chemical mismatch cleavage or by PCR-SSCP followed by DNA sequencing (19). No DNA sequence alterations were encountered in a total of 30 cases examined. Thus, the *TβR-II* gene is normally expressed in primary human breast cancer, and mutations of this gene are probably rare. This result is perhaps not surprising in light of previous studies of other cancer types: missense and/or nonsense mutations in the *TβR-II* gene have only been found sporadically in colorectal cancers, head-and-neck cancers, and cutaneous T-cell lymphomas (19, 20, 29, 30). The only exceptions are tumors that are associated with DNA mismatch repair deficiencies, which frequently display *TβR-II* nonsense mutations (31).

To determine whether mutations in the *TβR-I* gene might be found in human breast cancer, we screened each of the 9 exons of the *TβR-I* gene by PCR-SSCP in 31 primary breast carcinoma specimens and in 12 associated lymph node metastases. Areas of tumor tissue were isolated from paraffin sections by microdissection, and the remaining surrounding breast tissue was used to extract germline genomic DNA. Individual exons were amplified by PCR and the products screened for the presence of novel SSCPs. Suspect bands were reamplified and subjected to direct DNA sequencing. Individual sequence abnormalities were confirmed by repeating the entire procedure using a second aliquot of genomic DNA.

Our most important finding was a C to A transversion at nucleotide 1160 in exon 7 of *TβR-I*, which predicts for a serine to tyrosine substitution at codon 387 (Fig. 1). This was the only mutation encountered in the entire series and was present in 7 (16%, 95% CI, 7–31) of the 43 specimens (Fig. 1*A*). This is the first report of a mutation in the *TβR-I* gene in any type of human malignancy. Moreover, this mutation may be specifically associated with breast cancer inasmuch as we have not found it in any cervical carcinomas nor in head-and-neck cancer cell lines (32).[5] In addition, Pasche *et al.* (33) recently reported the absence of *TβR-I* mutations in acute myeloid leukemias.

Our second major finding is the highly significant association between the S387Y mutant and axillary lymph node metastases (Fig. 1*A*). Although we encountered this mutation in only 2 (6%, 95% CI, 1–21) of 31 primary breast cancer specimens, it was present in 5 (41%, 95% CI, 15–72) of the 12 lymph node metastases (Fisher's exact test, $P = 0.012$). The dramatically increased frequency of this mutation in lymph node metastases indicates that inactivation of the TGF-β pathway may represent a late event in breast cancer progression. The fact that most breast carcinoma cell lines are refractory to TGF-β is also consistent with this idea, inasmuch as most of these cell lines were initially derived from metastatic cells isolated from malignant pleural effusions or ascites (34). Moreover, in animal models of skin carcinogenesis, TGF-β-resistant tumor cell clones also do not emerge until the tumors have become highly aggressive and metastatic (35). In contrast, in colorectal cancers associated with DNA mismatch repair deficiencies, the acquisition of *TβR-II* gene mutations seems to coincide with the transition from preinvasive adenoma to invasive carcinomas (36, 37). Thus, the stage of tumor development at which the TGF-β signaling pathway becomes inactivated may vary depending on the tumor type and on the underlying molecular genetic events that drive the carcinogenetic process.

As the S387Y mutation was not detected in germline DNA of the same individuals (Fig. 1*B*), we can practically exclude the possibility that this sequence alteration represents a normal polymorphism. On the other hand, besides the mutant band, a wild-type band could be detected in each of the tumor specimens (Fig. 1*B*). Although these findings suggest that the tumors may have retained a wild-type allele, it is impossible to exclude the possibility that this wild-type band was the result of the almost inevitable contamination of the specimens with at least some normal cells. However, even the loss of function of one of the two *TβR-I* alleles may be sufficient to confer a significantly selective advantage. Such a dosage effect occurs, for example, in transgenic animals that express a dominant-negative *TβR-II* gene in conjunction with two endogenous wild-type alleles and in knockout mice that carry only a single *TGF-β1* gene allele (7, 9). In both of these situations, the animals are significantly more susceptible to tumor formation.

To test whether the serine to tyrosine substitution at position 387 disrupts receptor function, we introduced this mutation into a full-length wild-type *TβR-I* cDNA. We studied the effects of the mutation on receptor function in transient transfection assays using the TβR-I-deficient R-1B (L17) mink lung epithelial cell line. As shown in Fig. 2*A*, expression of wild-type *TβR-I* in R-1B (L17) cells resulted in an approximately 50% reduction in cyclin A promoter activity compared with cells transfected with an inert control vector. In contrast, pCAL2 activity was repressed by less than 30% in cells transfected with the S387Y receptor mutant (Fig. 2*A*). In cells transfected with wild-type *TβR-I*, pSBE4-dependent luciferase activity was increased approximately 15-fold over controls, whereas the increase observed in cells

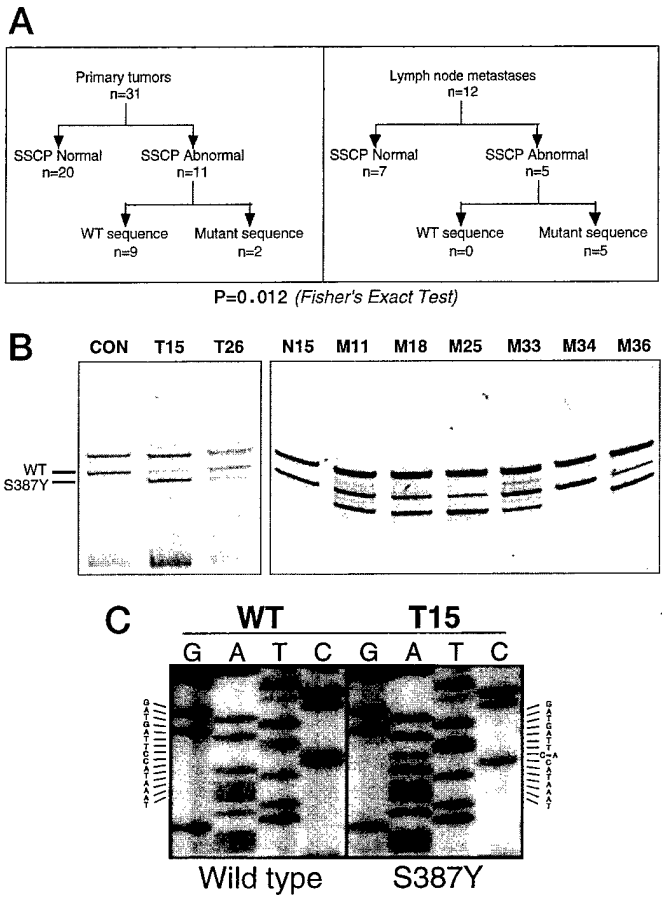

Fig. 1. Analysis of *TβR-I* gene exon 7 in human breast cancer specimens. In *A*, 19 stage I (axillary lymph node metastasis negative) and 12 stage II (axillary lymph node metastasis positive) breast carcinomas were analyzed for the presence of mutations within the *TβR-I* gene by PCR-SSCP and DNA sequencing. In *B*, SSCP analysis suggested the presence of a mutation in exon 7 in approximately one-half of the specimens. In *C*, the presence of a C to A transversion (nucleotide 1160), which predicts for a serine to tyrosine substitution at position 387 was confirmed by DNA sequencing in 7 (16%; 95% CI, 7–31%) of 43 specimens. The mutation was present in 2 (6%; 95% CI, 1–21) of 31 primary breast cancers as compared with 5 (41%; 95% CI, 15–72) of 12 lymph node metastases (Fisher's exact test, $P = 0.012$).

transfected with the receptor mutant was only approximately 10-fold (Fig. 2*B*). As shown in Fig. 2*C*, the S387Y mutation seems to induce a shift in the TGF-β dose-response relationship: wild-type *TβR-I*-expressing cells responded maximally to 50 pM TGF-β, whereas S387Y-expressing cells required at least 100 pM TGF-β for maximal response. Repression of the cyclin A promoter activity (pCAL2) correlates extremely well with the ability of cells to respond to TGF-β-mediated cell cycle arrest, and activation of the Smad DNA-binding element in pSBE4 reflects TGF-β-induced gene transcription (25, 26). To rule out that the observed differences in reporter gene activity were due to variations in levels of expression of wild-type and mutant *TβR-I* receptors in transfected cells, cell lysates were subjected to Western immunoblotting using anti-HA monoclonal antibody (Fig. 2*D*). Discrete M_r55,000 bands of equal intensity corresponding to the *TβR-I* receptor were detected in extracts from both wild-type and mutant *TβR-I*-transfected cells. Thus, the S387Y mutation did not affect receptor protein expression. In summary, cells expressing the S387Y mutant were significantly less sensitive to the effects of TGF-β on cell cycle regulation as well as transcriptional responses than cells expressing the wild-type receptor.

The exact mechanism whereby the S387Y mutation diminishes TGF-β signaling remains to be determined. According to the canonical domain subdivisions found in all protein kinases (38, 39), the

[5] V. F. Vellucci and M. Reiss, unpublished data.

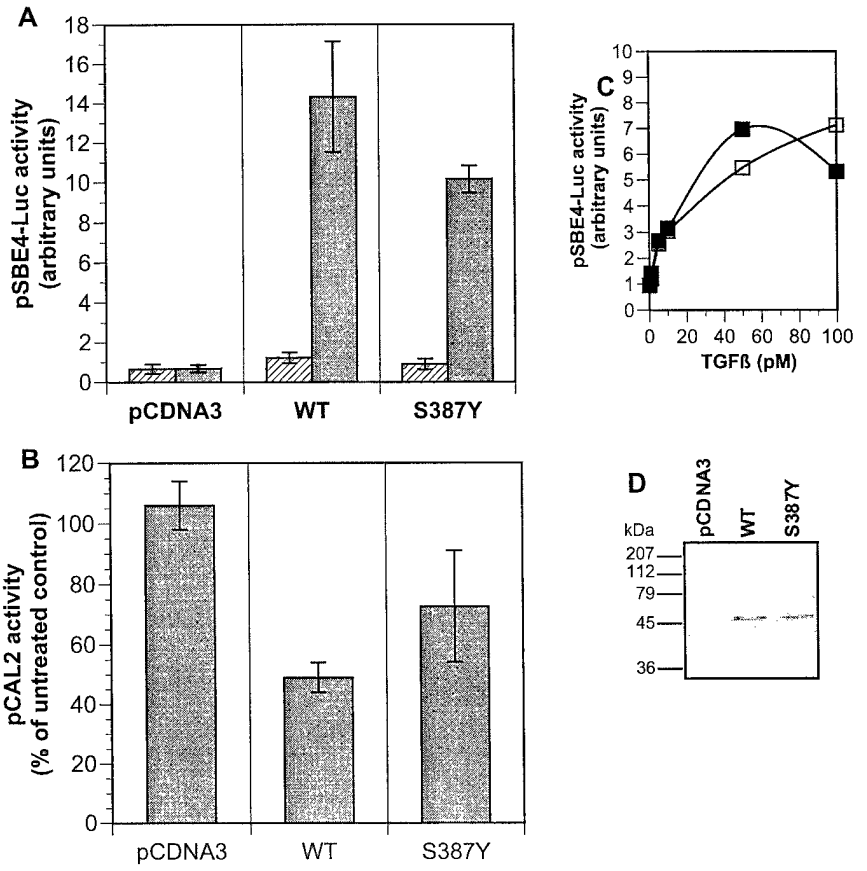

Fig. 2. The effects of transfected wild-type and mutant *TβR-I* receptors on TGF-β-regulated gene expression. R-1B (L17) cells were cotransfected with plasmids expressing either wild-type (*WT*) or mutant (*S387Y*) TβR-I receptor and pCAL2 (*A*) or pSBE4 (*B*) in conjunction with pRL-CMV, and luciferase activities in cell extracts were measured 48 h later as described in "Materials and Methods." Results were normalized for *Renilla* luciferase activity to correct for differences in transfection efficiency between experiments. In *A*, cyclin A promoter activity (*pCAL2*) was inhibited by 50% in cells transfected with the wild-type TβR-I, whereas cells transfected with the S387Y mutant expressed approximately 75% the amount of luciferase activity detected in control vector-transfected control cells. Means ± SE from 4 independent experiments. In *C*, in cells transfected with the TβR-I mutant (*S387Y*), TGF-β-induced SBE4-dependent luciferase activity (*pSBE4*) was increased to a significantly lesser extent than in wild-type TβR-I (*WT*) transfected cells. Means ± SE from four independent experiments. *D*, detection of wild-type (*WT*) and mutant (*S387Y*) TβR-I receptors in transfected R1-B (L17) cells by Western immunoblotting using HA.11 anti-HA antibody. Single discrete M_r 55,000 bands of equal intensity corresponding to the HA-tagged TβR-I receptor were detected in extracts from both wild-type and mutant TβR-I-transfected cells but not in control vector-transfected cells (*pCDNA3*).

serine residue at position 387 in TβR-I is located in the linker region between subdomains VIII and IX, which typically form the peptide recognition domain of protein kinases (38, 39; Fig. 3, *A* and *B*). The structures of subdomains VIII and IX are highly conserved among the family of type I TGF-β, activin, and BMP receptor serine-threonine kinases (Fig. 3*B*). The fact that these receptors share highly homologous substrates (Smads) further suggests that this region participates in substrate recognition. Alternatively, it may well affect the ho-

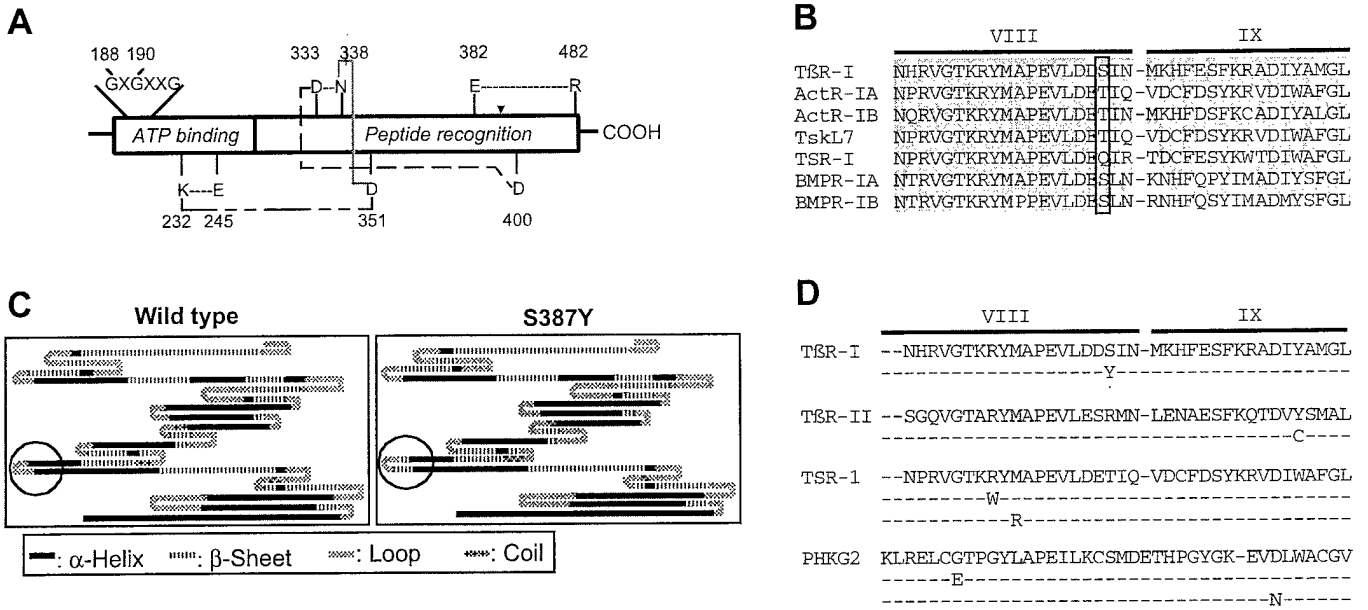

Fig. 3. *A*, structural features of TβR-I serine-threonine protein kinase catalytic domain, including the positions of amino acid residues that are highly conserved throughout the protein kinase superfamily (adapted from Taylor *et al.*; Ref. 38). ▼, position of S387Y mutation. *B*, comparison of amino acid sequence of peptide recognition domains (subdomains VIII and IX) of TGF-β, activin, and BMP type I receptor kinases *C*, predicted secondary structure of the wild-type and S387Y mutant TβR-I proteins based on their amino acid sequence using the algorithms described by Chou and Fasman (43). *D*, comparison of the location of S387Y mutant of TβR-I in breast cancer with mutations in *TSR-1* and *PHKG2* genes associated with hereditary hemorrhagic teleangiectasia type 2 and liver phosphorylase kinase deficiency syndromes, respectively (45–47), as well as in the *TβR-II* gene in head-and-neck cancer (30).

modimerization of TβR-I molecules or perhaps the interactions between TβR-I and -II molecules when they form heterotetrameric complexes during receptor activation.

The primary substrate of TβR-I, Smad2, is phosphorylated on two serine residues located within the consensus sequence $RCSS^{465}$-MS^{467} at the COOH terminus of the protein (40). Although the COOH-terminal tail of Smad2 is not absolutely required for its physical interaction with TβR-I, structure-function studies indicate that it clearly plays a complimentary role in enzyme-substrate recognition and partly determines specificity between TGF-β and BMP signaling (41). Comparison between the crystal structures of activated protein kinase A in complex with an inhibitory peptide and that of the TβR-I kinase indicates that the S387Y residue does not fall precisely within the canonical substrate binding site as defined in the protein kinase A-inhibitory peptide structure (42). However, this does not exclude the possibility that the substitution of a tyrosine with its larger side chain for the serine at position 387 in TβR-I interferes with productive substrate recognition, particularly as the interface between a Smad and TβR-I is probably much larger than the protein kinase A-inhibitory peptide interface.[6] Furthermore, on the basis of the Chou and Fassman algorithm (43), one would predict that the S387Y mutation alters the secondary structure of the TβR-I kinase by introducing two β-sheets flanking the loop that connects the E and F α-helices of the catalytic core (Fig. 3*C*). It is worth noting that two other TGF-β type I receptors, TSR-1 and TskL7, contain different polar residues at position 387 (threonine and glutamine, respectively; Fig. 3*B*). Interestingly, neither of these two receptors is able to elicit the same cellular responses as TβR-I, perhaps because they are unable to interact with Smad2 or -3 (44).

Finally, the functional importance of this region is also illustrated by the fact that several syndromes have been associated with mutations within subdomains VIII or IX in other protein kinases. For example, two different arginine to tryptophan and methionine to arginine mutations in the *TSR-1* gene have been described in hereditary hemorrhagic telangiectasia type 2 (Fig. 3*D*; Refs. 45, 46). Moreover, amino acid substitutions at highly conserved glutamate and aspartate residues in the catalytic subunit of phosphorylase B kinase result in the loss of enzyme activity, glycogenosis, and liver cirrhosis (47). In addition, Wang *et al.* (30) recently described a case of head-and-neck cancer with a tyrosine to cysteine mutation within subdomain IX of the TβR-II serine-threonine kinase. Although the effects of this mutation on receptor function were not reported in this case, it is likely that it affects enzyme activity as well.

In summary, we have identified a single missense mutation of the *TβR-I* gene that occurs with relatively high frequency in invasive ductal breast cancer and that has a significant negative impact on receptor signaling. This is the first reported missense mutation in this gene reported in any human malignant neoplasm and provides further support for the idea that inactivation of the TGF-β signaling pathway can play an important role in human carcinogenesis. Furthermore, the high frequency of the S387Y mutation in lymph node metastases suggests that inactivation of this signaling pathway may be particularly associated with the metastatic phenotype.

Acknowledgments

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[6] M. Huse and J. Kuriyan, personal communication.

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Breast Cancer Research and Treatment **45**: 81–95, 1997.

Hypothesis

Transforming growth factor-β in breast cancer: A working hypothesis

Michael Reiss and Mary Helen Barcellos-Hoff
Department of Medicine (Medical Oncology), Yale University School of Medicine, New Haven, CT (MR) and Radiation Biology & DNA Repair, Life Sciences Division, UC/Ernest O. Lawrence Berkeley National Laboratory, Berkeley, CA (MHB-H), USA

Key words: breast cancer, TGFβ, activation, resistance, receptors, tamoxifen, chemoprevention

Summary

Transforming Growth Factor-β (TGFβ) is the most potent known inhibitor of the progression of normal mammary epithelial cells through the cell cycle. During the early stages of breast cancer development, the transformed epithelial cells appear to still be sensitive to TGFβ-mediated growth arrest, and TGFβ can act as an anti-tumor promoter. In contrast, advanced breast cancers are mostly refractory to TGFβ-mediated growth inhibition and produce large amounts of TGFβ, which may enhance tumor cell invasion and metastasis by its effects on extracellular matrix. We postulate that this seemingly paradoxical switch in the responsiveness of tumor cells to TGFβ during progression is the consequence of the activation of the latent TGFβ that is produced and deposited into the tumor microenvironment, thereby driving the clonal expansion of TGFβ-resistant tumor cells. While tumor cells themselves may activate TGFβ, recent observations suggest that environmental tumor promoters or carcinogens, such as ionizing radiation, can cause stromal fibroblasts to activate TGFβ by epigenetic mechanisms. As the biological effects of the anti-estrogen tamoxifen may well be mediated by TGFβ, this model has a number of important implications for the clinical uses of tamoxifen in the prevention and treatment of breast cancer. In addition, it suggests a number of novel approaches to the treatment of advanced breast cancer.

Introduction

Because the components of the molecular machinery that controls the cell cycle are often mutated in human neoplasia, cancer may be considered a disorder of the cell cycle [1]. However, whether or not a given cell actually enters the cycle and proceeds through cell division is critically dependent on the input it receives from growth factors and growth inhibitors in the extracellular milieu. One of these factors, Transforming Growth Factor-β (TGFβ), is the most potent physiological inhibitor of cell cycle progression of normal epithelial cells, such as those in the mammary gland [2, 3]. During mammary gland development, TGFβ selectively inhibits ductal elongation by causing the disappearance of the proliferating stem cell layer and rapid involution of ductal end buds, while alveolar morphogenesis is not affected [4, 5]. Moreover, transgenic expression of TGFβ1 targeted to the mammary epithelium inhibits the normal development of the ductal and lobular epithelium in a dose-dependent manner [6,

Address for offprints and correspondence: Michael Reiss, Department of Internal Medicine, Section of Medical Oncology, Yale University School of Medicine, 333 Cedar Street, Box 208032 CT 06520-8032, New Haven, USA; *Tel:* 203-785-6221; *Fax:* 203-785-6221; *e-mail:* MichaelReiss@quickmail.yale.edu

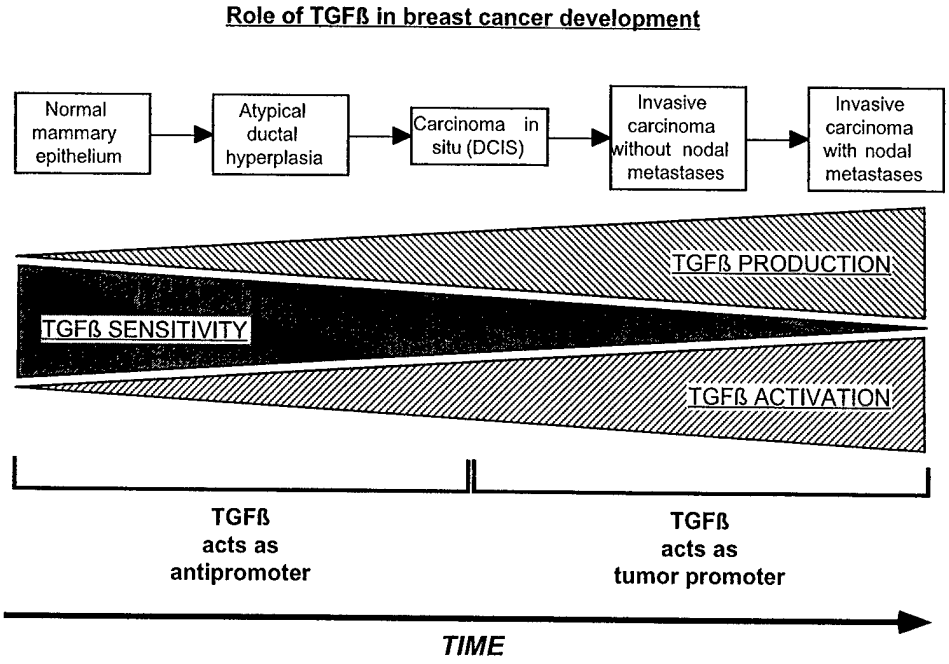

Figure 1. Proposed role of TGFβ in mammary carcinogenesis. Human breast cancers at successive stages of tumor progression appear to be associated with increasing production of TGFβ, suggesting that this growth factor is providing a selective advantage. In parallel, one sees the preferential outgrowth of TGFβ-resistant tumor cell populations, which is driven by the activation of the TGFβ present in the tumor microenvironment.

7]. In the adult mammary gland, TGFβ appears to control the massive cell death and restructuring that takes place during post-lactational involution [8]. Thus, TGFβ is a critical regulator of the temporal and spatial patterns of epithelial cell proliferation and regression that take place during mammary gland development and during and after lactation.

Based on these physiological effects, it is not surprising that TGFβ has also been implicated in mammary carcinogenesis. One view that has been proposed is that TGFβ functions primarily as a growth inhibitor for breast cancer cells, and mediates the cytostatic and chemopreventive actions of anti-estrogens, such as tamoxifen (reviewed in [9]). An alternative view is that breast cancer cells produce TGFβ which somehow promotes tumor progression, while the tumor cells themselves are refractory to TGFβ-mediated cell cycle arrest (reviewed in [10]).

We would like to propose that these divergent views are both compatible with the notion that the role of TGFβ undergoes a shift during breast cancer progression from being predominantly an anti-promoter during the early stages of neoplasia to becoming conducive to cancer invasion, and perhaps metastasis, by advanced tumors (Figure 1). We propose that this shift is brought about by the increasing production and release of TGFβ by the tumor cells, the activation of latent TGFβ within the microenvironment, and the clonal expansion of tumor cells that are resistant to TGFβ on the basis of inactivation of genes encoding TGFβ receptors or perhaps other elements of the signaling pathway. This working hypothesis reconciles the two seemingly contrary views of TGFβ's role in breast cancer and has important consequences for chemoprevention as well as therapy of the disease.

Production of TGFβ by breast cancer cells increases during neoplastic progression

The TGFβs (TGFβ1–3) comprise a family of highly conserved dimeric 25 kDa polypeptides that are ubiquitously expressed in normal mammalian tis-

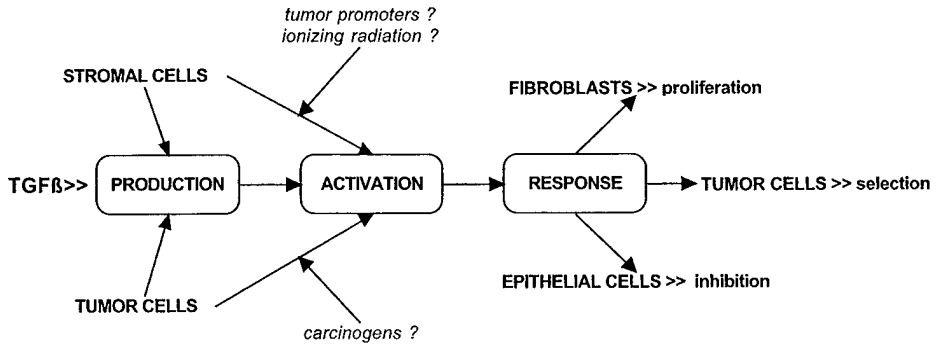

Figure 2. Putative relationship between TGFβ production, activation, and biological effects in invasive breast cancer. Either the epithelial tumor cells themselves or surrounding stromal fibroblasts can be the source of tumor associated TGFβ. Similarly, activation of latent TGFβ can be caused by genetic mutations acquired by the tumor cells, or by epigenetic events that affect the surrounding stromal cells, such as, for example, exposure to carcinogens, tumor promoters, or ionizing radiation. Activated TGFβ acts as a mitogen for normal fibroblasts, and may be responsible for the desmoplastic reaction often seen in breast carcinoma, while it provides a negative selective force that favors the expansion of TGFβ-resistant epithelial tumor cell clones.

sues [11]. TGFβs exert two major biological effects on epithelial cells. First, picomolar amounts of TGFβ are able to arrest human mammary epithelial cells (HME) at the G_1/S boundary, resulting in complete inhibition of DNA replication and clonal growth [2, 3]. Secondly, TGFβ elicits a series of cellular responses that include, for example, the induction of fibronectin and other protein components of extracellular matrix, as well as plasminogen activator inhibitor type 1 [12, 13]. This second set of responses results in a net accumulation of extracellular matrix.

That neoplastic transformation and progression of human breast epithelial cells might be associated with an increased constitutive production of TGFβ was first suggested by tissue culture models. For example, 184 HME cells and oncogene-transformed sublines are exceedingly sensitive to TGFβ-mediated growth arrest, and secrete barely detectable amounts of TGFβ [3]. In contrast, most cell lines derived from invasive human breast carcinomas secrete much larger amounts of TGFβ, mostly TGFβ2 [3, 14, 15]. In primary human breast cancers, TGFβ is localized in and around the epithelial tumor cells, while the surrounding stromal cells are negative [16–18]. Even more striking is the observation that the production of TGFβ by primary breast cancers appears to increase with advancing stages of tumor progression. For example, Walker and Dearing [16] reported that 45% of carcinomas *in situ* and 66% of invasive carcinomas contained immunodetectable amounts of TGFβ, whereas there was no staining of adjacent normal epithelium. Moreover, the strongest staining was observed in invasive carcinomas with associated lymph node metastases. These results have been confirmed independently by several other studies [17–19]. Interestingly, most of the TGFβ is deposited at the advancing edges of the tumors in areas of active growth, suggesting a possible role in tumor cell invasion [18]. This increased production of TGFβ in human breast cancer associated with tumor progression suggests that it may be acting as a tumor promoter rather than as an inhibitor of tumor growth.

There are several different ways by which tumor-derived TGFβ might promote tumor progression. One possibility is that TGFβ affects cell-cell and/or cell-substrate interactions, resulting in a greater propensity for invasion and/or metastasis. For example, human breast cancer cells that had been exposed to TGFβ *in vitro* or had been transfected with a TGFβ1 expression vector were significantly more tumorigenic when they were injected into nude mice than control cells [20, 21]. Another possibility is that tumor-derived TGFβ1 acts as an immune suppressor, and allows the tumor cells to escape from immune surveillance [22–24].

Table 1. Effects of TGFβ on proliferation of normal and transformed epithelial cells of the mammary gland–summary of published studies

Species	Cell line(s)	Transforming agent or tumor type	Assay used	IC_{50} (pM)	Maximal inhibition	Concentration (pM)	Ref.
Man	Primary human mammary epithelial cells (HMEC)	None	ADG	8–36	80–100%	120	[2]
Man	184A1 HMEC (immortalized)	Benzo(a)pyrene	ADG	10	70–100%	40	[2]
Man	184B5 HMEC (immortalized)	Benzo(a)pyrene	ADG	10–> 800	20–100%	40–800	[2]
Man	Primary human mammary epithelial cells	None	ADG	3.2	75%	100	[3]
Man	A1N4 HMEC (immortalized)	Benzo(a)pyrene	ADG	2.6	44.3%	100	[3]
Man	A1N4T HMEC (immortalized)	SV40 (large T)	ADG	1.2	86%	100	[3]
Man	A1N4M HMEC (immortalized)	v-*mos*	ADG	0.7	63%	100	[3]
Man	A1N4H HMEC (weakly tumorigenic)	v-Ha-*ras*	ADG	1.1	78.3%	≥ 10	[3]
Man	A1N4MH HMEC (weakly tumorigenic)	v-*mos* + v-Ha-*ras*	ADG	1.5	82.7%	100	[3]
Man	A1N4TH HMEC (highly tumorigenic)	SV40 (large T) + v-Ha-*ras*	ADG	2.9	33.7%	100	[3]
Man	MCF-7A	Adenocarcinoma (ER positive)	AIG	NA	86%	100	[47]
Man	MCF-7B	Adenocarcinoma (ER positive)	AIG	NA	36%	100	[47]
Man	LY2	Adenocarcinoma (ER positive)	AIG	NA	28%	100	[47]
Man	MDA-MB-231	Adenocarcinoma (ER negative)	AIG	NA	93%	100	[47]
			ADG	NA	86.5%	100	
Man	MCF-7	Adenocarcinoma (ER positive)	ADG	> 100	10%	100	[14]
			TI	> 1000	No inhibition	1000	
			AIG	> 100	No inhibition	100	
Man	MCF-7L	Adenocarcinoma (ER positive)	ADG	> 100	10%	100	[14]
			TI	> 1000	No inhibition	1000	
			AIG	> 100	No inhibition	100	
Man	T47D	Adenocarcinoma (ER positive)	ADG	> 100	No inhibition	100	[14]
			TI	> 1000	No inhibition	1000	
Man	ZR75-1	Adenocarcinoma (ER positive)	ADG	> 100	20%	100	[14]
			TI	> 1000	20%	1000	
			AIG	> 100	20%	100	
Man	MDA 330	Adenocarcinoma (ER negative)	ADG	> 100	10%	100	[14]
			TI	> 1000	40%	1000	
			AIG	> 100	45%	100	
Man	BT20	Adenocarcinoma (ER negative)	ADG	> 100	30%	100	[14]
			TI	> 1000	10%	1000	
			AIG	2	80%	100	
Man	MDA-MB-231	Adenocarcinoma (ER negative)	ADG	100	50%	100	[14]
			TI	100	60%	1000	
			AIG	0.5	100%	100	

Activation of TGFβ in the tumor microenvironment

As indicated above, the malignant progression of breast cancer appears to be associated with the increased production and secretion of TGFβ by the tumor cells themselves. However, the active form of TGFβ1 is derived from a 390-amino acid precursor which is processed into a homodimer of the mature 112 amino acid carboxy-terminal TGFβ1 peptide in a non-covalent association with a dimer of the processed N-terminal pro-segment, called the latency-associated peptide (LAP). This latent TGFβ complex is secreted but unable to bind to TGFβ receptors unless the biologically active mature TGFβ is dissociated from the LAP [25]. Since TGFβ receptors are apparently ubiquitously expressed [26] and latent TGFβ is abundant in all tissues [11], release from the latent complex is the key control of TGFβ's biological activity. This so-called activation is considered the critical event that regulates TGFβ function *in vivo* [27]. TGFβ activation in normal adult tissue *in vivo* appears to be the principal switch that initiates the response to damage and orchestrates the acute inflammatory reaction. Thus, TGFβ has been shown to play a prominent role in wounding, ischemia/reperfusion injury, the response to ionizing radiation, and other causes of acute inflammation (reviewed in [28]). Under these physiological conditions, the effects of TGFβ activation are rapidly limited by the production of antagonists, such as decorin. Consequently, elevated

Table 1. Continued

Species	Cell line(s)	Transforming agent or tumor type	Assay used	IC_{50} (pM)	Maximal inhibition	Concentration (pM)	Ref.
Man	HS578T	Adenocarcinoma (ER negative)	ADG TI	100 5	50% 85%	100 1000	[14]
Man	MCF-7	Adenocarcinoma (estrogen dependent)	TI	200	58%	400	[49]
Man	MCF-7	Adenocarcinoma (estrogen independent)	TI	400–1000	65%	1000	[49]
Man	MDA-MB-231	Adenocarcinoma (ER negative)	AIG	4	75%	40	[48]
Man	SK-BR-3	Adenocarcinoma (ER negative)	AIG	20	75%	200	[48]
Man	Hs578T	Adenocarcinoma (ER negative)	AIG	4	75%	40	[48]
Man	MDA-MB-468	Adenocarcinoma (ER negative)	AIG	20	100%	400	[48]
Man	MDA-MB-468-S4	Adenocarcinoma (ER negative)	AIG	> 400	No inhibition	400	[48]
Man	MCF-7	Adenocarcinoma (ER positive)	ADG	40	55%	80	[15]
Man	T47D	Adenocarcinoma (ER positive)	ADG	> 80	No inhibition	80	[15]

Summary of published studies illustrating the differential sensitivity to the anti-proliferative effect of TGFβ between non-neoplastic HMEC and human breast cancer cells *in vitro* as well as *in vivo*. The average IC_{50} of TGFβ for breast cancer cells is considerably higher and the maximally achievable inhibition of growth consistently lower than for normal HMEC. This difference is of the same order of magnitude as that found between other types of primary epithelial cells and the carcinomas derived from them (see [10] for review). Immortalization of primary HMEC is not associated with the acquisition of TGFβ-resistance, but the development of tumorigenic (i.e. invasive) properties is, suggesting that there is a linkage between these two phenotypic characteristics of breast carcinomas. ADG : Anchorage-dependent growth; ER: Estrogen receptor; AIG: Anchorage-independent growth; NA: Not available; TI: Thymidine incorporation.

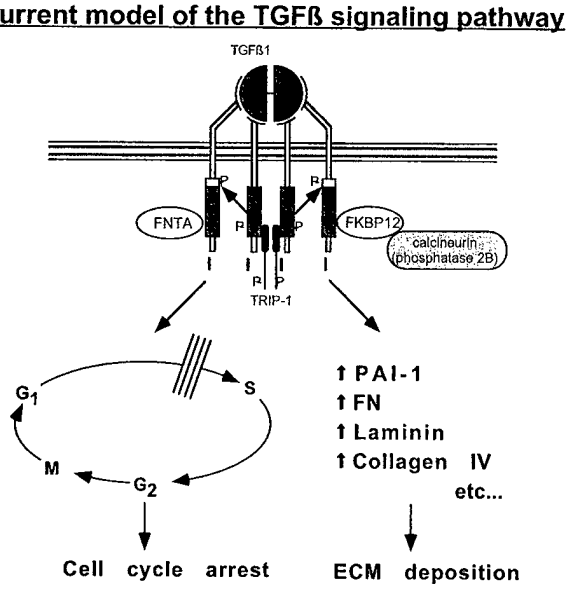

Figure 3. Current model of TGFβ signaling pathway. TGFβ1 binds to TβR-II homodimers followed by the recruitment of one (or more) TβR-I receptor molecules into a stable ternary complex with TβR-II, which results in the phosphorylation of the GS domain of TβR-I by the TβR-II kinase. The α-subunit of the *ras* farnesyltransferase (FNTA) and FKBP-12 are associated with TβR-I. FKBP-12 functions as a bridge molecule between TβR-I and the Ca^{2+}/calmodulin-dependent protein phosphatase 2B (calcineurin), while FNTA is involved in activation of the *ras* pathway. The function of the TβR-II-associated protein TRIP-I is unknown. TGFβ induces the expression of cyclin-dependent kinase inhibitors, such as $p15^{INK4B}$ and $p21^{WAF-1}$, whereas it inhibits expression of cyclins A and E, as well as cdk4, presumably leading to cell cycle arrest in mid- to late G_1. In addition, TGFβ induces the expression of genes that encode extracellular matrix proteins as well as plasminogen activator inhibitor-1.

expression of latent complex by itself (as seen in tumor tissue) is unlikely to have major biological consequences unless it is accompanied by increased activation (Figure 2) [29].

It is possible that tumor cells themselves cause the activation of the TGFβ that they produce. *In vitro*, a significant fraction of the TGFβ that breast carcinoma cells secrete into the culture medium is in its activated form [3, 14, 15]. Whether breast carcinoma cells also cause activation of TGFβ *in vivo* is not clear because none of the published immunohistochemical studies employed antibodies that specifically recognize the activated form of the TGFβ. However, advanced breast cancers often produce increased amounts of urokinase-type plasminogen activator (uPA) [30–32], which catalyzes the conversion of plasminogen to plasmin. Because plasmin is believed to activate latent TGFβ, the increased levels of uPA in tumors may cause TGFβ activation [33].

The recent observation that stromal cells are the principal source of breast cancer-associated uPA is compatible with the notion that fibroblasts and not tumor cells induce TGFβ activation [34]. Alternatively, environmental agents may cause TGFβ activation by stromal cells within the tumor microenvironment, such as occurs during the acute response to tissue injury. For example, we have shown recently that ionizing radiation leads to a rapid and global remodeling of the microenvironment in the virgin mouse mammary gland [35]. Within 24 hr of exposure to doses of 5 Gy or less, we observed loss of collagens type I and III in the peri-epithelial stroma and *de novo* expression of collagen type III in the adipose stroma [35]. In addition, hyaluronic acid was lost in the irradiated mammary gland and the glycoprotein tenascin was induced at the stromal/epithelial interface (M.H. Barcellos-Hoff, unpublished data). Both tenascin and hyaluronic acid have been implicated in epithelial migration during tissue remodeling and wound healing [36, 37]. Moreover, tenascin is developmentally regulated in mouse mammary gland and is normally turned off in adult tissue [38]; however it is re-expressed in malignant breast tumors of both mice and humans, suggesting that its anti-adhesive characteristics may be conducive to tumor growth and invasion [38–40].

It is striking that the proteins that are induced by radiation are all known to be regulated by TGFβ. Using antibodies that discriminate between the active and latent forms of TGFβ [41], we found that latent TGFβ was abundant in the normal mammary gland, but that active TGFβ was restricted to epithelial structures, where it was faintly detectable. However, at 1 hr after radiation exposure, latent TGFβ immunoreactivity was strikingly diminished in the adipose stroma, while active TGFβ immunoreactivity was induced in the previously negative adipose stroma and dramatically increased within the epithelium and peri-epithelial stroma. These data suggest that, either directly or indirectly, ionizing radiation induces TGFβ activation. This pattern persisted in the epithelium for 24 hr, and did not re-

vert to pre-irradiation conditions in the stroma for at least 7 days. Furthermore, TGFβ activation was detected at doses as low as 0.1 Gy and failed to show a threshold effect (E.J. Ehrhart and M.H. Barcellos-Hoff, submitted). Besides ionizing radiation, chemical tumor promoters have also been shown to activate TGFβ. For example, phorbol ester treatment of carcinogen-initiated skin rapidly induces TGFβ mRNA and protein immunoreactivity [42, 43] that most likely reflects the production of active TGFβ, since the antibody used in these studies reacts specifically with active TGFβ [41, 44]. Similarly, phenobarbital induces active TGFβ immunoreactivity in normal liver tissue, resulting in the selective clonal expansion of transformed hepatocytes that express decreased levels of TGFβ receptors [45, 46].

The observations that radiation and chemical agents can induce the activation of a cytokine that is instrumental in restraining growth may appear paradoxical. This paradox may be resolved by assuming that TGFβ activation causes a selective expansion of cells in which mutations confer resistance to TGFβ, as is observed, for example, in diethylnitrosamine-induced liver tumors [46]. Conversely, one would predict that transformation in which TGFβ activation does not occur would not be associated with TGFβ-resistance of the associated tumors. Thus, carcinogenic agents such as radiation appear to have a dual role: Besides their classic carcinogenic effect of inducing mutations in the target cell DNA, such agents also fundamentally alter the microenvironment in which the damaged epithelial cells reside (e.g. by causing the activation of latent TGFβ), thereby promoting the selective outgrowth of mutant cells that display a particular phenotype (in this case, TGFβ-resistance). The recognition of these epigenetic effects of radiation may have important implications for the therapeutic use of ionizing radiation, as will be discussed below.

Effects of TGFβ on breast carcinoma cells

In vitro, mammary epithelial cell lines range from being exquisitely sensitive to being completely refractory to TGFβ-mediated growth inhibition (Table 1). For instance, spontaneously immortalized HMEC (A1N4 cells) are nearly as sensitive to TGFβ as primary HMEC, even when stably transfected with single viral oncogenes [3]. In contrast, only highly tumorigenic variants of A1N4, obtained by transfection with the combination of v-Ha-*ras* and SV40 large T antigen, are significantly less responsive to TGFβ than their non-tumorigenic precursors [3]. Moreover, compared with HMEC, most cell lines derived from invasive human breast carcinomas are much less sensitive to the anti-proliferative effects of TGFβ, depending, to some extent, on whether cells are grown in monolayer or in soft-agar. By and large, TGFβs appear to be more potent inhibitors of anchorage-independent growth than of growth in monolayer culture [14, 47, 48], perhaps as a consequence of their effects on cell-matrix interactions rather than a direct effect on cell cycle progression.

Several studies have suggested that estrogen-receptor (ER)-negative breast cancer cell lines are relatively more sensitive to TGFβ than ER-positive ones [47, 48], although there is considerable variability between studies. For example, clonal growth in monolayer culture of the ER-negative line MDA-MB-231 was found to be strongly inhibited by TGFβ in two studies [47, 48], but not in a third [14]. Another possibility is that TGFβ-sensitivity is primarily a function of the estrogen-dependence of the breast cancer cells, rather than the expression of hormone receptors. For example, estrogen-dependent MCF-7 breast cancer cells are quite sensitive to TGFβ-mediated growth inhibition, whereas estrogen-independent sublines are refractory to TGFβ [49]. In any event, even if modest differences in TGFβ sensitivity exist among breast carcinoma cell lines, the average IC_{50} of TGFβ for tumor cells is considerably higher and the maximally achievable inhibition of growth consistently lower than for primary HMEC (Table 1).

In vivo, transgenic mice that produce a constitutively active form of TGFβ1 have been found to be resistant to 7,12-dimethylbenz[a]anthracene-induced mammary tumor formation [50]. Furthermore, cross-breeding of such mice with a strain that overexpressed the epithelial mitogen TGFα, in which mammary tumors develop at a high rate, also resulted in marked reduction in the incidence of

mammary tumors [50]. On the other hand, treatment of mice bearing MDA-MB-231 human breast cancer xenografts with TGFβ did not result in any suppression of tumor growth *in vivo* [51]. In aggregate, these studies provide compelling evidence that overexpression of TGFβ1 can markedly suppress *de novo* mammary tumor development, but that this effect is lost once invasive carcinomas have arisen.

Mechanisms of escape from TGFβ control

In order to understand the mechanisms that cause tumor cells to become refractory to TGFβ-mediated cell cycle arrest, it is necessary to examine the molecular components of the TGFβ signaling pathway. The biological effects of TGFβ1 are transduced by two interacting and interdependent receptor subtypes, type I (TβR-I) and type II (TβR-I) (Figure 3) [52, 53]. Both are highly conserved transmembrane serine-threonine receptor kinases [54–56]. Binding of TGFβ1 to TβR-II homodimers is followed by the recruitment of TβR-I receptor molecules into a stable heterotetrameric complex with TβR-II (Figure 3) [57]. This triggers the phosphorylation of the juxtamembrane (GS) domain of TβR-I by the TβR-II kinase [58, 59]. The activated TβR-I kinase presumably phosphorylates downstream elements in the signaling cascade [59].

A number of proteins which physically interact with TβR-I [60] or with the TβR-II receptor [61], may be required for signaling. For example, FKBP-12 functions as a bridge molecule between TβR-I and the Ca^{2+}/calmodulin-dependent protein phosphatase 2B (calcineurin) [60, 62]. Moreover, the α-subunit of the *ras* farnesyltransferase (FNTA), which is also associated with TβR-I, is phosphorylated and released upon TGFβ stimulation, indicating that activation of *ras* and the mitogen-activated protein kinase cascade may play a role in mediating the cellular responses to TGFβ [63–66]. Further downstream, TGFβ induces the expression of the cyclin-dependent kinase inhibitors $p15^{INK4B}$ and $p21^{WAF-1}$ [67, 68], whereas expression of cyclins A and E as well as cdk4 is inhibited [69, 70].

Thus far, molecular alterations of only two cell cycle control genes have been associated with loss of responsiveness to TGFβ. We and others [71, 72] have shown that cells that express mutant forms of the p53 tumor suppressor protein lose sensitivity to TGFβ. Secondly, Okamoto et al. [73] noted TGFβ-resistance in esophageal epithelial cells overexpressing cyclin D1. The p53 gene is often mutated in human breast cancers and cyclin D1 levels are elevated in one third to one half of all breast cancers [74–76]. In a tumor microenvironment that contains activated TGFβ, even a partial loss of responsiveness due to expression of mutant p53 or an excess of cyclin D1 in the tumor cells may well confer a selective proliferative advantage.

The main mechanism that results in complete abrogation of TGFβ responsiveness is inactivation of either one of the two TβR receptors [58, 77]. There appears to be a direct quantitative relationship between the level of TβR-II expression in tumor cell lines and TGFβ-responsiveness. For example, somatic cell fusion of two different, TGFβ-refractory, TβR-II-negative carcinoma cell lines gave rise to hybrids that re-expressed TβR-II protein and regained sensitivity to TGFβ [78]. Several TGFβ-resistant human breast cancer cell lines fail to express TβR-II mRNA transcripts [52, 79]. Moreover, transfection with a TβR-II expression vector results in the restoration of TGFβ-responsiveness *in vitro* and, most importantly, in suppression of tumorigenicity *in vivo* [79, 80]. Besides TβR-II, genetic defects of the TβR-I receptor can also result in TGFβ resistance: Kim et al. [81] recently identified a human prostatic carcinoma cell line that was refractory to TGFβ because of loss of TβR-I expression as a consequence of gene rearrangement. The relationship between loss of TβR expression and TGFβ resistance appears to hold up *in vivo* as well. For example, Kadin et al. [82] demonstrated that the loss of TβR-II transcripts parallels the progressive loss of TGFβ responsiveness in cells obtained from serial biopsies of a single patient with progressive $Ki\text{-}1^+$ cutaneous T-cell lymphoma. Similarly, malignant CD4+ lymphocytes obtained from patients with Sézary syndrome are also refractory to TGFβ and fail to express TβR-II cell surface receptors [83].

The importance of loss of TβR-II expression in

primary human solid tumors remains to be established. We showed recently that TβR-II transcripts are absent in approximately 25% of primary esophageal carcinomas [84]. Moreover, TβR-II receptor levels appear to be reduced in high-grade prostatic carcinomas [84]. On the other hand, primary malignant melanomas also express normal TβR-II mRNA levels [85], while TβR-II transcripts appear to be increased in pancreatic carcinomas compared to adjacent normal tissue [86]. Thus far, each of the primary breast carcinomas that we screened expressed TβR-II transcripts (L. Garrigue-Antar and M. Reiss, unpublished observations). Thus, although changes in TβR-II gene expression do occur in human tumors, there is no evidence as yet that this occurs in primary breast cancer.

Besides loss of TβR-II expression, we recently identified several mutants of the TβR-II receptor gene in human squamous head-&-neck carcinoma cell lines that account for their resistance to TGFβ1-mediated cell cycle arrest [87]. Mutational inactivation of the TβR-II gene also occurs frequently in colorectal carcinoma cell lines as well as in primary colorectal and gastric cancers from patients with an inherited DNA mismatch repair deficiency [88–91]. Although mutations within the TβR-I gene have not yet been described, it is likely that they occur in human cancers as well.

At this point, structural information about TGFβ genes in primary human breast cancers is extremely limited. Ke et al. [92] were unable to detect any TβR-II microsatellite mutations in a small series of sporadic breast carcinomas. However, in our own preliminary analysis of primary breast cancer specimens, we have encountered several TβR-II missense mutations which are likely to affect receptor kinase function (L. Garrigue-Antar and M. Reiss, unpublished observations). Thus, genetic alterations of the TβR-II gene do occur during human mammary carcinogenesis, but the actual frequency of such events still remains to be established.

Clinical implications

The experimental data summarized above indicate that the malignant progression of breast cancer is associated with the increased autocrine production, secretion, and activation of TGFβ. While, in the early stages of breast cancer development, mammary epithelial cells are sensitive to growth inhibition by TGFβ, TGFβ-resistance and the accompanying higher levels of TGFβ production by the tumor cells probably represent late events in breast cancer progression, associated with a greater invasive and/or metastatic potential (Figure 1).

This model has a number of important implications with respect to the chemoprevention and treatment of breast cancer. For example, therapeutic maneuvers designed to induce and/or activate TGFβ would be expected to have their greatest therapeutic benefit in the setting of a breast carcinoma or a preneoplastic lesion that is still sensitive to its anti-proliferative effect. On the other hand, treatments that induce the production and/or activation of TGFβ may be associated with the risk of enhancing growth or invasiveness of tumors that have arisen in a microenvironment that contains activated TGFβ. In these cases, targeting TGFβ itself might be a more effective therapeutic strategy, as has been proposed for the treatment of chronic inflammatory conditions and of malignant gliomas [24, 93].

Particularly intriguing is the possibility that the clinical and chemopreventive effects of tamoxifen may be mediated by TGFβ. Treatment of human ER-positive breast cancer cell lines with tamoxifen *in vitro* induces the production and secretion of TGFβ by these tumor cells and slows down their growth [47]. This observation suggested that the antitumor effects of tamoxifen on established invasive and metastatic cancer might be mediated by TGFβ, and that this was dependent on the expression of functional ER. Paradoxically, ER-negative breast cancer cell lines appear to be more sensitive to TGFβ-mediated growth inhibition than ER-positive ones, at least *in vitro* [14]. Moreover, some breast cancer cell lines respond to tamoxifen in spite of the fact that they are refractory to TGFβ-mediated growth arrest because they do not express TGFβ receptors [15, 80, 94]. These *in vitro* studies may be confounded by the low responsiveness of all cell lines derived from established breast carcinomas to TGFβ compared to normal HMEC, and by

the fact that *in vitro* assays fail to take into account the role of stromal cells in mediating tumor growth *in vivo*. In this light, it is interesting that a number of recent clinico-pathological studies have demonstrated that tamoxifen induces the production of TGFβ isoforms *in vivo*: Thus, several studies have demonstrated elevations of plasma levels of TGFβ2 in response to tamoxifen treatment, and these elevations appear to correlate with tumor regression [95, 96]. In addition, a number of investigators have examined the effects of tamoxifen treatment on the expression of TGFβ isoforms in breast cancer specimens. Although some of these studies suggested that TGFβ1 was induced in tumor stroma in response to tamoxifen [97], others have failed to confirm these results [98, 99]. However, several recent reports seem to indicate that the predominant TGFβ isoform induced in breast cancer-associated stromal fibroblasts following tamoxifen treatment is TGFβ2 [99, 100]. Although none of these studies formally prove that TGFβ mediates the effects of tamoxifen on breast tumor growth, they strongly suggest a relationship between the induction of TGFβ2 in tumor stroma with elevations of TGFβ2 plasma levels, and tumor regression.

The key point here is that, *in vivo*, tamoxifen-responsiveness may be dependent not so much on whether or not the breast cancer cells express hormone-receptors, but rather on whether or not they are still sensitive to TGFβ-mediated growth arrest. Although the majority of ER-positive breast cancers respond to tamoxifen, a significant number do not. For example, approximately 30% of women with ER-positive tumors who are treated with tamoxifen in the adjuvant setting eventually suffer recurrences, indicating that these tumors are tamoxifen-resistant [101]. Furthermore, 40–45% of ER-positive metastatic tumors are also clinically resistant to anti-estrogen therapy [102]. Tamoxifen-resistance may be the result of a decreased expression of ER [103], mutations in the ER or PgR genes [104], or the increased conversion of tamoxifen to inactive metabolites [105]. However, in a significant proportion of cases, none of these mechanisms is operative [103]. We would like to propose that some ER-positive tumors are resistant to tamoxifen because the tumor cells no longer respond to TGFβ-mediated cell cycle arrest. This may also explain why primary breast cancers that are clinically resistant to tamoxifen express the highest levels of TGFβ1 [106]. Moreover, tumors that express low levels of uPA are more likely to responsed to tamoxifen than those that express high uPA, in which TGFβ is presumably activated [107]. Consequently, uncovering the molecular basis of TGFβ resistance should allow us to better identify patients who are likely to benefit from anti-estrogen therapy, as well as those for whom tamoxifen may be detrimental.

Based on our model, one would predict that treatments that activate TGFβ would be most effective when applied early on in breast cancer progression. For example, in addition to causing cell kill by inducing DNA damage, the particularly strong beneficial effects of ionizing radiation in the treatment of *in situ* breast cancer may be mediated by TGFβ activation and TGFβ-mediated apoptosis [108, 109]. Similarly, there is good rationale for the ongoing clinical studies of tamoxifen in the treatment of *in situ* breast cancer. Because the pharmacological induction of TGFβ expression and activation might also be able to prevent primary breast cancer [110], tamoxifen is currently being evaluated as a chemopreventive agent in large scale clinical trials [111, 112]. Similarly, the chemopreventive actions of certain retinoids may be mediated by the activation of TGFβ in stromal elements [113, 114]. Monoterpenes, such as d-limonene, also represent potentially useful chemopreventive agents in breast cancer [115]. In rodent models, these agents have been shown to increase the expression of mannose 6 phosphate/insulin-like growth factor-II receptors (M6P/IGF-IIR) and TGFβ1 by mammary carcinoma cells [115]. Expression of M6P/IGF-IIR facilitates the activation of TGFβ, thereby restoring the autocrine growth inhibitory loop and preventing the outgrowth of transformed cell clones.

Conversely, in the treatment of advanced TGFβ-resistant breast cancers, tamoxifen may be ineffective and perhaps may even promote tumor growth. Similarly, resistance of tumor cells to TGFβ may negate some of the cytotoxic effects of radiation treatment, and radiation-dependent activation of TGFβ in the stroma may even be detrimental if it makes the microenvironment more conducive to tumor

progression and contributes to normal tissue damage leading to fibrosis [116]. This is perhaps the reason that radiation appears to be more effective in providing local control after resection of small, mammographically detected tumors than of larger, palpable lesions. In these types of settings, one should consider therapeutic strategies aimed at inhibiting the activity of TGFβ. In fact, radiation-induced TGFβ may even be detrimental in the case of a TGFβ-sensitive breast tumor, as TGFβ-dependent cell cycle arrest may allow for repair of DNA damage. Two different approaches to prevent inflammation-associated fibrosis by inactivating TGFβ have been successfully tested in experimental models [28, 93]. These include the use of neutralizing antibodies to TGFβ [117] and the administration of the TGFβ-binding protein, decorin [118]. Based on the idea that tumor-derived TGFβ allows tumors to escape from immune surveillance, a recent study showed that regression of experimental brain tumors in rats could be achieved by expressing antisense TGFβ2 mRNA by enhancing immune rejection [24]. Similar approaches might be developed for the treatment of advanced breast cancers that are associated with TGFβ activation.

In summary, the detection of activated TGFβ in breast cancer stroma and/or the identification of molecular lesions in the TGFβ signaling system may provide new clinical tools to distinguish tumors in which augmentation of TGFβ production and/or activation might be therapeutically beneficial from those in which this would not be the case. This is particularly pertinent for the identification of patients who are likely not to benefit from anti-estrogen or radiation therapy. Conversely, the development of agents aimed at inactivating TGFβ is likely to benefit the treatment of advanced breast cancer, as has been suggested for the treatment of chronic inflammatory conditions associated with fibrosis [93].

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